

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵: A01N 63/00, A61K 48/00, A01K 49/00, C12P 21/06, C12N 5/00, 5/16, 5/22, 15/00, 7/01	A1	(11) International Publication Number: WO 94/24870 (43) International Publication Date: 10 November 1994 (10.11.94)
(21) International Application Number: PCT/US94/00650 (22) International Filing Date: 17 January 1994 (17.01.94) (30) Priority Data: 08/006,478 5 May 1993 (05.05.93) US (60) Parent Application or Grant (63) Related by Continuation US 08/006,478 (CIP) Filed on 20 January 1993 (20.01.93) (71) Applicants (for all designated States except US): BIO- TRANSPLANT, INC. [US/US]; Building 96, 13th Street, Charlestown Navy Yard, Charlestown, MA 02129 (US). THE GENERAL HOSPITAL CORPORATION [US/US]; Fruit Street, Boston, MA 02114 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LE GUERN, Christian, A. [FR/US]; 140 Dickerman Road, Newton Highlands, MA 02161 (US). SEED, Brian [US/US]; 9 Hawthorne Place, SJ, Boston, MA 02114 (US). BANERJEE, Papia, T. [US/US]; 67 Ward Street, Lexington, MA 02173 (US).		(74) Agent: HERRON, Charles, J.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US). (81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: RETROVIRAL VECTORS CAPABLE OF EXPRESSING MULTIMERIC PROTEINS FROM MULTIPLE TRANSLATIONAL INITIATION SITES (57) Abstract This invention relates to retroviral vectors that express multiple polypeptide subunits of a eukaryotic protein from a single polycistronic mRNA and the proteins produced from these vectors. The expressed proteins are particularly useful for inducing transplantation tolerance and the vectors are useful as agents for mediating gene transfer into eukaryotic cells.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

RETROVIRAL VECTORS CAPABLE OF EXPRESSING MULTIMERIC
PROTEINS FROM MULTIPLE TRANSLATIONAL INITIATION SITES

This application is a continuation-in-part of copending U.S. serial no. 08/006,478, filed January 20, 1993.

This invention relates to retroviral vectors that express multiple polypeptide subunits of a eukaryotic protein from a single polycistronic mRNA and the proteins produced from these vectors. The expressed proteins are particularly useful for inducing transplantation tolerance.

Retroviral vectors are useful as agents to mediate gene transfer into eukaryotic cells. These vectors are generally constructed such that the majority of sequences coding for the structural genes of the virus are deleted and replaced by the gene(s) of interest. The retroviral vector contains the signals required for packaging of the virus but does not contain the information required for the generation of an infectious virus particle. The retroviral vector (in the form of a plasmid DNA) is transfected into a packaging cell line, which is capable of supplying, in trans, the retroviral gene product(s) necessary to generate a virus particle. The packaging cell line is incapable of giving rise to a retrovirus in the absence of the retroviral vector. Virus produced by the transfected packaging cell line may be used to infect other cells, including retroviral packaging cell

lines and hematopoietic cells. The retroviral vector sequences may be integrated into the host genome via retroviral mediated integration and the genes of interest contained within the retroviral vector may be stably expressed off the integrated proviral form. It is necessary that the packaging cell lines are generated in such a fashion as to minimize the potential for recombination between the retroviral structural genes and the sequences present in the retroviral vector. If such recombination were to occur infectious retroviral particles might be generated which could replicate in any host cell.

The genes of interest may be incorporated into the proviral backbone in several general ways. The most straightforward constructs are ones in which the structural genes of the retrovirus are replaced by a single gene which is then transcribed under the control of the viral regulatory sequences within the long terminal repeat (LTR). Other vectors contain promoters in addition to the viral promoter contained in the 5' LTR.

Recently, retroviral vectors have been designed to include the use of internal promoter elements to initiate transcription. One potential problem with retroviral vectors containing multiple transcription units is that, if selection is applied for one gene, expression of the other gene can be reduced or lost completely. This has been termed "promoter suppression" (Emerman & Temin, 1986, Nuc. Acids Res., 14: 9381-9396). This phenomenon of promoter suppression is the reason that the most recent literature reports in the field have concluded that multiple gene expression from a single transcript is preferred (Adam, M. A. et al., 1991, J. Virol, 65: 4985-4990; Ghattas, I.R. et al., 1991, Mol. Cell. Biol. 11: 5848-5859 and Morgan, R.A. et al., 1992, Nuc. Acids. Res. 20: 1293-1299).

Gene expression of multiple genes from a single eukaryotic

transcript was first observed in the picorna viral mRNAs. It has been well documented that the 5' untranslated sequence of these viral mRNAs contains a sequence which acts as an internal ribosome entry site (IRES) and which functions to facilitate protein translation from sequences located downstream from the first AUG of the mRNA. Using a picorna viral IRES sequence three genes have been expressed from a single construct, in which one of the genes is transcriptionally driven by the SV40 promoter and the remaining two by the retroviral LTR promoter (Adam et al., supra). In one instance two IRES sequences were used in one single transcript (Morgan, et al., supra.).

Macejak and Sarnow, ((1991) Nature, 353: 90-94) reported that the 5' untranslated sequence of the immunoglobulin heavy chain binding protein (BiP, also known as GRP 78, the glucose-regulated protein of molecular weight 78,000) mRNA can directly confer internal ribosome binding to an mRNA in mammalian cells, in a 5'-cap independent manner, indicating that translation initiation by an internal ribosome binding mechanism is used by this cellular mRNA.

This invention represents the first use of the BiP IRES sequence in a retroviral vector. It also represents the first use of more than two IRESs for translation initiation from a single retroviral transcript, as well as the first use of multiple copies of the same IRES in a single construct. Further, it provides the potential, for the first time, to independently express 4 to 5 polypeptides from a single retroviral construct. The invention further makes possible the production of multiple polypeptides by a single retroviral construct that are capable of forming a single, functional, multimeric protein. A particularly valuable embodiment of this invention provides the first instance of this type of retroviral approach to express heterodimeric major histocompatibility proteins on the cell surface. In particular, the

vectors may be used to express xenogeneic proteins for the specific purpose of inducing transplantation tolerance in potential transplant recipients.

The retroviral vectors of the invention have been constructed to have several 5' untranslated regions (UTR) derived from either eukaryotic cellular DNA or viral sequences that act as internal ribosome binding sites for multiple translation initiations from a single eukaryotic transcript. The 5' untranslated regions preferred in accordance with the invention may be multiple copies of a 210 base pair fragment corresponding to the 5' untranslated region of human immunoglobulin heavy-chain binding protein (BiP) mRNA. Alternatively, the vectors may include IRES sequences that are derived from picorna viral sequences as well as the BiP IRES sequence. The vectors are capable of producing several polypeptides that, when posttranslationally linked form, functional heteromultimeric proteins. The proteins produced with the vectors of the invention are particularly those useful for inducing transplantation tolerance, including several different major histocompatibility complex (MHC) heterodimeric proteins. The invention also provides a process for constructing these retroviral vectors.

Abbreviations are: LTR - retroviral long terminal repeat; BiP - IRES sequence from the human immunoglobulin chain binding protein; GPT - sequence of the E. coli gpt gene that encodes the enzyme xanthine-guanine phosphoribosyl transferase; CAT - sequence of the E. coli gene that encodes the enzyme chloramphenicol acetyl transferase; Neo - sequence of E. coli Tn5 gene that encodes the enzyme neomycin phosphotransferase; DR α and DR β - mini-swine MHC II DR α and DR β (c haplotype) cDNA genes; EMC - the IRES sequence from encephalomyocarditis virus. Transfected GP/E-envAM12 cells are distinguished by the prefix AM, while transfected GP/E-86 cells are distinguished by the prefix GP. Transduced PA317 cells with virus

generated from GP/E-86 cells are identified by the prefix GPA.

Figure 1 graphically illustrates the retroviral vector pL7gCAT and each of vectors pPBM1 through pPBM5, and pPBM7 through pPBM10, each of which is briefly described as follows:

pL7gCAT: This retroviral vector is a derivative of pLNCX (Miller and Rosman, 1989, *Biotechniques*, 7: 980-990) and is capable of expressing three different genes under the translational control of three BiP IRES. This vector also contains two reporter genes which encode chloramphenicol acetyl transferase (CAT) and xanthine-guanine phosphoribosyl transferase (GPT).

pPBM1: The 3'LTR of M-MLV present in pL7gCAT has been replaced by the 3'LTR of M-MPSV (murine myeloproliferative sarcoma virus).

pPBM2: In order to achieve detectable transient expression of the genes from COSM6 cells, a copy of the enhancerless SV40 origin of replication was inserted before the 3'LTR at the ClaI site, thereby generating pPBM2. A unique BglII site was created during the construction of pPBM2. pPBM2 is designed to express GPT and CAT.

pPBM3: A copy of the mini-swine MHC class II DR_β cDNA gene (Gustafsson et al. 1990, *J. Immunology*, 145: 1946-1951) was inserted in pPBM2 after the first BiP IRES, between the HindIII and NotI sites. pPBM3 is designed to express DR_β, GPT and CAT.

pPBM4: A polylinker sequence was inserted at the EcoRI site in pPBM3. This modification potentiates the introduction of another gene which would be translated from the 5'LTR. The EcoRI site was destroyed during this modification. pPBM4 is designed to express DR_β, GPT and CAT.

pPBM4-prime: The orientation of the SV40 ori fragment was reversed from its orientation in pPBM4. During the construction of pPBM4-prime the polylinker sequence was eliminated from the vector, the EcoRI site was re-created and one of the ClaI sites was destroyed.

pPBM5: The neomycin resistance gene was inserted downstream of the second copy of the BiP IRES sequence, between the NotI and BglII sites of pPBM4. pPBM5 is designed to express DR β and the neomycin resistance gene.

pPBM7: The mini-swine MHC class II DR α cDNA was inserted after the second BiP IRES sequence in the NotI-BglII site of pPBM4. pPBM7 was designed to express the mini-swine MHC class II cDNA genes for DR α and DR β .

pPBM8: pPBM7 was modified to include a third BiP IRES sequence and the neomycin resistance gene. pPBM7 was designed to express the mini-swine MHC class II cDNA genes for DR α and DR β , together with the neomycin resistance gene.

pPBM9: A 2.8 kb fragment was generated by PCR using the primers HindIII-linked 5' DR β (SEQ. ID. NO: 7) and ClaI-linked-3'SV40ori (SEQ. ID. NO: 5), utilizing pPBM8 as template. The fragment was inserted into HindIII and ClaI digested pPBM4.

pPBM10: pPBM10 is identical to pPBM9 except that the SV40 ori has been eliminated. A 2.62 kb fragment was generated by PCR using primers HindIII-linked 5' DR β (SEQ. ID. NO: 7) and ClaI-linked 3' Neomycin (SEQ. ID. NO: 19) and pPBM8 as a template. The DNA fragment was inserted into HindIII and ClaI digested pPBM4.

Figure 2 shows a schematic diagram of the protocol used to construct pPBM1 from pL7gCAT. In this case the ClaI-SacI fragment

of pL7gCAT was exchanged with that of pMPZen (Johnson et. al., 1989, EMBO J., 8: 441-448) in order to replace the MoMLV 3'LTR of pL7gCAT with a hybrid 3'LTR that contains the transcriptional promoter enhancer region of Myeloproliferative Sarcoma Virus (M-MPSV). J1, J2, J3 and J4 indicate the oligonucleotide primers used in polymerase chain reactions and correspond to SEQ. ID. NO: 1, SEQ. ID. NO: 2, SEQ. ID. NO: 3 and SEQ. ID. NO: 4, respectively.

Figure 3 shows an autoradiograph of a thin layer chromatography plate depicting the results of an assay for the activity of the chloramphenicol acetyl transferase (CAT assay). In this assay, Rous Sarcoma Viral promoter-driven expression of the CAT gene (pRSVcat - ATCC 37152) is the positive control for demonstrating the conversion of ¹⁴C-labeled chloramphenicol to its acetylated monomeric form. All other vectors are as described in the text.

Figure 4 shows a bar graph demonstrating the amount of neomycin phospho-transferase polypeptide produced per ml of the COSM6 cell lysates following transient expression of the retroviral construct, pPBM5. Neo-pl and Neo-flask refer to experiments demonstrating that pPBM5, containing the neomycin phosphotransferase gene, when transfected into COSM6 cells grown on a tissue culture plate (Neo-pl) or in a tissue culture flask (Neo-flask), respectively, expressed high levels of neomycin phosphotransferase. RSV-pl stands for a similar transfection with pRSV-CAT which was used as the negative control.

Figure 5 shows a flow cytometry analysis detailing the expression of mini-swine MHC class II molecules following transient transfection of COSM6 cells.

A Contemporaneous histograms from a flow cytometry analysis of COSM6 cells transfected with pPBM4 which does not contain the DR α sequence. No apparent shift in fluorescence intensity is observed

when the cells were stained with the MHC class II specific antibody, ISCR3, as compared to the fluorescence intensity of the cells stained with isotype control antibody 74-12-4.

B Contemporaneous histograms from COSM6 cells transfected with pPBM7. The higher fluorescence intensity of staining of the cells with the ISCR3 antibody is indicated by the shift of the histogram obtained with ISCR3 as compared to that obtained with 74-12-4, the isotype control antibody.

Figure 6 is a flow chart summary for the experiments reported in Example 2.

Figure 7 shows a flow cytometry analysis of the surface expression of the mini-swine MHC class II DR α / β heterodimer from integrated polycistronic retroviral constructs.

The histograms were obtained by staining with the antibody 40D which specifically recognizes the DR α / β heterodimer and with 76-2-11 antibody which is specific for IgG2b and which is the isotype control for 40D.

(A) and (B) - the staining patterns of untransfected GP/E-86 and GP/E-envAM12 cells respectively.

(C) and (D) - the staining patterns of a mixed population ("bulk") of G418 resistant sub-clones of GP/E-86 and GP/E-envAM12 respectively, that were transfected with pPBM8.

(E) and (F) - the staining patterns of cells that had been transduced with a mixture ("bulk") of sub-clones; (E) - packaged virus from transfected GP/E-envAM12 cells were used to transduce the GP/E-86 cell line, described as GP>AM bulk; (F) - packaged virus from transfected GP/E-86 cells had been used to transduce the GP/E-envAM12 cell line, described as AM>GP bulk .

Figure 8 shows a flow cytometry analysis of individual G418

resistant sub-clones obtained after transfection with pPBM8. In each panel the histogram obtained by staining with 40D antibody was superimposed on that obtained with 76-2-11. The cells were also stained with an MHC Class I antibody, B1094. (A) and (C) are the histograms obtained from untransfected GP/E-86 and GP/E-envAM12 cell lines, respectively. (B) is the histogram of the subclone GP1²-2 which was derived by transfection of GP/E-86 cells with pPBM8. (D) is the histogram of the subclone AM1¹_a-6 which was derived by transfection of GP/E-envAM12 cells with pPBM8.

Figure 9 shows a photograph of an agarose gel showing the DNA fragment produced by polymerase chain reaction using a primer specific for the 5' end of porcine DR β cDNA, HindIII-linked 5' DR β (SEQ. ID. NO. 7) in conjunction with a primer specific for the 3' end of porcine DR α cDNA, BglII-linked 3'DR α (SEQ. ID. NO. 16). The template DNAs used for the PCR analysis were obtained from individual retroviral sub-clones. lane 1 is λ -BstEII DNA marker; lane 2 is DFK cells, a negative control (Hirsch, F. et. al., 1992. J. Immunol. 149: 841-846) ; lane 3 is AM1¹_a-6; lane 4 is AM1¹_a-4; lane 5 is AM1¹_c-3; lane 6 is GP2³-1; lane 7 is GP9¹_a-3; and lane 8 is GP1²-2.

Figure 10 shows the Southern blot analysis of the chromosomal DNAs from the transfected G418 resistant producer cell lines which were generated using pPBM8.

(A) shows an autoradiograph in which the respective names of the transfected cell lines are indicated at the top of each lane. The radioactively labeled probe was a randomly primed porcine DR β cDNA fragment. The sizes of the DNA fragments were estimated by comparing the mobilities of the bands to those of λ BstEII DNA markers. The estimated size of the DNA fragments in each lane is indicated by the arrow.

(B) is a schematic representation of the retroviral insert in pPBM8. Sizes of DNA fragments which might have been generated

through potential deletional events occurring as a consequence of recombination between the BiP sequences are indicated.

Figure 11 shows a graphical illustration for each of vectors pPBM13 and pPBM14. pPBM13 is designed to express the DR β sequence under the translational control of the 5'LTR and the DR α sequence under the translational control of the BiP IRES sequence. pPBM14 is identical to pPBM13 except for the inclusion of the neomycin resistance gene which is under the control of the EMCV IRES.

Figure 12 shows a flow cytometry analysis of the expression of the mini-swine MHC class II DR α / β heterodimer on the surface of COSM6 cells transiently transfected with pPBM13. The histogram was obtained by staining the cells with antibody 76-2-11 (an isotype control), antibody W362 (specifically recognizes monkey class I) or antibody 40D (specific for MHC class II DR α / β heterodimer).

Figure 13 shows flow cytometry analyses of the expression of the mini-swine MHC class II DR α / β heterodimer on the surface of GP/E-86 cells. (A) GP/E-86 cells which had been transfected with pPBM5 which does not contain the sequences enabling DR α / β heterodimer expression. (B) - (D) show histograms for individual G418 resistant clones, generated by transfection of GP/E-86 cells with pPBM14.

Figure 14 shows flow cytometry analyses of sub-clones derived from PA317 cell line transduced with a transiently expressed viral supernatant from GP/E-86 cells, transfected with pPBM14. (A) is a flow cytometry analysis of PA317 cells transduced with the supernatant obtained from mock-transfected GP/E-86 cells. (B)-(F) are flow cytometry analyses for individual transduced G418 resistant sub-clones.

Figure 15 shows a bar graph detailing the percentage of cells expressing the MHC class II DR α / β heterodimer on the surface at any

given time, relative to the isotype control. The name of each subclone is as indicated on the abscissa of the bar graph.

Figure 16 shows the results from slot blot hybridization experiment of RNA isolated from the supernatants of G418 resistant sub-clones. (A) Blot hybridized with randomly primed radioactively labeled DR α probe. (B) Blot hybridized with randomly primed radioactively-labeled DR β probe. The names of the sub-clones are as indicated.

In one aspect, the invention provides retroviral vectors comprising (i) a single transcription regulatory sequence at the 5' region thereof; (ii) a first DNA coding sequence; (iii) at least one additional DNA coding sequence; and (iv) an IRES sequence controlling the translation of each such additional DNA coding sequence of (iii), wherein the vector expresses multiple independent polypeptides capable of posttranslational combination to form at least one functional multimeric protein. The retroviral vectors produce, for example, a functional heterodimeric protein of the major histocompatibility complex.

In a preferred embodiment of this aspect, the IRES is a BiP IRES. Such vectors where the IRES is a BiP IRES preferably produce a single heterodimeric protein of the major histocompatibility complex. Exemplary vectors are selected from the group of pL7gCAT, pPBM1 through pPBM14 and derivatives of any of them ("derivative" refers to structurally modified but substantially functionally equivalent constructs). Also contemplated are cells containing the retroviral vectors of this aspect, which cells are preferably mammalian (particularly human).

Another aspect of the invention provides a retroviral vector comprising (i) a single transcription regulatory sequence at the 5' region thereof; (ii) a first DNA coding sequence; (iii) at least two additional DNA coding sequences; and (iv) IRESs controlling the

translation of each such additional coding sequence to express at least one functional multimeric protein, wherein at least one of such IRESs is a cellular IRES. Preferably, at least one such cellular IRES is a mammalian IRES (e.g., a BiP IRES) and the other IRESs can be viral IRESs, (e.g., encephalomyocarditis IRES, a polio virus IRES or a hepatitis virus IRES).

In another aspect, the invention provides a cell or tissue made to contain the retrovirus of the invention. The cell is preferably human.

In another aspect, the invention provides a method for inducing immune tolerance in a human recipient for graft transplantation which comprises reintroducing, into said human recipient, cells explanted from said human and made to contain a vector of the invention. Preferably the DNA coding sequences code for polypeptides of a functional heterodimeric protein of the major histocompatibility complex of a non-human. Preferably the non-human is porcine (e.g., miniature swine) or primate.

The protein product of the vector(s) of the invention is a functional protein formed of multiple polypeptide subunits that are combined by bridging moieties, or "linkers", e.g., disulfide bridges.

In one embodiment, for inducing donor specific tolerance in a transplant recipient candidate, the vectors of the invention are constructed to express both chains of at least one heterodimeric major histocompatibility complex protein.

In a particularly preferred embodiment of the present invention, a polycistronic retroviral vector has been constructed which utilizes one copy of the 210 base pair 5' untranslated sequences from Human Immunoglobulin Heavy Chain Binding Protein

(BiP). This expresses three polypeptides from a single transcript, all utilizing the BiP sequence. Also, one more expressible gene can be put under direct control of a 5' LTR and yet another under control of the SV40 promoter-enhancer.

In a particularly preferred embodiment of the present invention, a polycistronic retroviral vector has been constructed which utilizes one copy of the 210 base pair 5' untranslated sequence from Human Immunoglobulin Heavy Chain Binding Protein (BiP) and one copy of the encephalomyocarditis IRES. A third polypeptide is expressed under the translational control of the retroviral 5' LTR. This vector expresses three polypeptides from a single transcript, two of which post-translationally combine to form a multimeric protein of the major histocompatibility complex. Also, one more expressible gene can be put under direct control of the SV40 promoter-enhancer.

The major advantages in using the BiP sequence are (i) its lack of secondary structure; (ii) absence of any internal AUG sequences; (iii) small size for viral packaging purposes; and (iv) most of all the ability to direct translational initiation in from all three positions irrespective of the downstream distance from the 5'LTR.

An additional novel aspect of this retroviral vector is the use of a hybrid 3' LTR in which the U5 sequence was taken from Murine Proliferative Sarcoma Virus (M-MPSV), and the R and U3 sequences were taken from M-MLV, because it had been shown previously that M-MPSV bears a very strong transcriptional enhancer which drives over-expression in myeloid tissues. (Bowtell, D.D.L. et. al., 1988. J.Virol. 62: 2464-2473) .

Examples of retroviral vectors which may be used include vectors derived from Moloney Murine Leukemia Virus, Spleen Necrosis

virus, Rous Sarcoma Virus and Harvey Sarcoma Virus. Specific vectors which may be constructed in accordance with the present invention are described in the Examples.

EXAMPLE 1

CONSTRUCTION AND ANALYSIS OF POLYCISTRONIC RETROVIRAL VECTORS

Construction of pL7gCAT

The retroviral vector LNCX described in Miller and Rosman, 1989, Biotechniques, 7: 980-990 was modified by eliminating the neomycin phosphotransferase gene and the CMV promoter and converting it to the polycistronic vector, pL7gCAT.

Construction of pPBM1

In this construction (Figure 1) the goal was to replace M-MLV 3'LTR from pL7gCAT with the 3'LTR from Murine Myeloproliferative Sarcoma virus (M-MPSV) which bears a relatively strong enhancer-promoter for transcription in cells derived from myeloid tissue. This manipulation was done at the 3'LTR region because the 3'LTR is duplicated during the retroviral integration process and thereby functions as the 5'LTR transcriptional enhancer-promoter from the integrated proviral state. A PCR fragment (Mullis, K.B. & Faloona, F.A., 1987, Methods Enzymol., 155: 335-350; Saiki, R.K. et.al., 1988, Science: 239: 487-491) was generated using two oligonucleotide primers, termed J1 (SEQ. ID. NO: 1) and J2 (SEQ. ID. NO: 2) and the plasmid pMPZEN (Johnson et. al., 1989, EMBO J., 8: 441-448). The primer J1 corresponds to nucleotides 2102 through 2119 of M-MPSV (Genbank accession number K01683). The primer J2 corresponds to the reverse complement of nucleotides 2693 through 2709 of the M-MPSV sequence. The 608 base pair (bp) PCR fragment was digested with restriction endonucleases ClaI and SacI, electrophoresed through an agarose gel; the 551 bp ClaI/SacI DNA fragment was excised and isolated using the Geneclean II DNA purification kit (Bio 101 Inc., La Jolla, CA). pL7gCAT was also

digested with ClaI and SacI, electrophoresed through an agarose gel. The PCR fragment that had been purified with the Geneclean II solution was ligated to ClaI/SacI digested pL7gCAT. The ligation mixture was used to transform E. coli JM109 (ATCC 53323). Positive sub-clones were identified by colony hybridization using a ³²P-labeled 104 bp PCR generated fragment using pMPZEN and J3 (SEQ. ID. NO: 3) and J4 (SEQ. ID. NO: 4) as the PCR primers. J3 corresponds to nucleotides 2127 - 2143 and J4 to the reverse complement of nucleotides 2215 - 2231 of M-MPSV. The clone pPBM1 was subjected to DNA sequence analysis, using J1 as primer to confirm the presence of the M-MPSV sequence in pPBM1.

Analysis of CAT expression from pL7gCAT and pPBM1

COSM6 cells (a transformed African Green Monkey Kidney cell line) were transfected (Aruffo, A. and Seed, B., 1987, Proc. Natl. Acad. Sci. U.S.A. 84: 8573- 8577) with pPBM1 and pL7gCAT and tested for CAT expression (Gorman et. al., 1982, Mol. Cell Biol. 2: 1044-1051; Prost. E. and Moore, D., 1986, Gene 45: 107-111). CAT activity was not detectable from transfections using either of the two plasmids. There were two possible explanations for the lack of CAT expression:- (1) that the preliminary DNA sequence analysis of pL7gCAT was incorrect and (2) that, since neither vector contained the SV40 ori, the lack of gene expression was a consequence of the inability of the vectors to undergo DNA replication in COSM6 cells. DNA sequence analysis of pL7gCAT and pPBM1 revealed that, while the initiator methionine and the following sequence were intact in the CAT gene, there was a deletion of 11 nucleotides between the 3' junction of the third BiP sequence and the CAT gene. In order to establish whether or not those 11 nucleotides were essential for internal translational initiation, the SV40 ori sequence was inserted into pPBM1. This vector is described as pPBM2.

Construction of pPBM2.

A PCR fragment was generated using ClaI-linked-3' SV40ori (SEQ.

ID. NO: 5) and ClaI-BglIII-linked-5'SV40ori (SEQ. ID. NO: 6) primers with pCDM8 plasmid DNA (Seed, B., 1987, Nature 329: 840-842) as the template. The PCR fragment was cleaved by ClaI and gel purified. The ClaI-digested PCR fragment was subcloned in the ClaI site of pPBM1. In this construct, the SV40ori sequence went in the orientation such that the BglIII site is located adjacent to the 3'LTR (See Figure 1: pPBM2).

Analysis of CAT expression from pPBM2

COSM6 cells were transfected with pPBM2 and analyzed for the production of CAT. The transient expression of CAT harvested from COSM6 cells transfected with pPBM2 was comparable to the level of CAT produced by cells transfected with the control plasmid pRSVCAT (Figure 3). These results indicate that the deletion of the 11 nucleotides from pL7gCAT did not result in the lack of apparent expression of the CAT gene in vectors pL7gCAT and pPBM1. Rather, the lack of expression was a consequence of the inability of these vectors to replicate in COSM6 cells.

Construction of pPBM3: The next vector in the series of vectors which would ultimately result in a vector capable of expressing the mini-swine MHC class II DR α / β cDNA genes was one which contained the mini-swine MHC class II DR β cDNA gene. The DR β cDNA-containing plasmid pPBSKSII-DR β (Gustafsson, K. et. al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 9798-9802; Shafer, G.E. et. al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88: 9760-9764) was used as a template, in a PCR reaction in the presence of primers HindIII-linked 5'DR β (SEQ. ID. NO: 7) and NotI-linked 3' DR β (SEQ. ID. NO: 8). The fragment was cleaved with HindIII and NotI and inserted between the HindIII and NotI sites of pPBM2. The vector generated is described as pPBM3. Analysis of the transient expression of the CAT gene from pPBM3 (Figure 3) indicated that the insertion of the DR β cDNA sequence did not affect the level of CAT expression, relative to pPBM2.

Construction of pPBM4 and pPBM4-prime

A polylinker sequence containing BstXI, SspI and MluI restriction sites was created by annealing two 25 bp long complementary oligonucleotides poly1 (SEQ. ID. NO: 9) and poly2 (SEQ. ID. NO: 10) and inserting the fragment into the EcoRI site in pPBM3, thereby generating pPBM4. DNA sequence analysis of pPBM4 revealed that three copies of the polylinker were present in the plasmid. For subsequent constructions it was necessary to change the orientation of the SV40 ori such that the BglII site would be located at the 5' end of the SV40 ori fragment. Hence, the SV40ori fragment was excised and re-subcloned in ClaI digested pPBM4 vector, generating pPBM4-prime. During the construction of pPBM4-prime the polylinker was eliminated, the EcoRI site was re-created and the ClaI site at the 3' end of SV40ori sequence was destroyed.

Construction of pPBM5: A BiP IRES-containing DNA fragment was generated from pL7gCAT by PCR using the primer NotI-linked 5' primer (SEQ. ID. NO: 11), which contains the nucleotides 376 - 400 of the human GRP78 sequence (Genbank Accession number M19645) and a NotI site, and primer EcIR (SEQ. ID. NO: 12), which contains the reverse complement to nucleotides 569-587 of the human GRP78 sequence (Genbank Accession number M19645) and an EcoRI site. The PCR fragment was cleaved with NotI and EcoRI and purified by gel electrophoresis and GeneCleanII. A neomycin phosphotransferase resistance gene-containing DNA fragment was generated by PCR using primer EXneo (SEQ. ID. NO: 13), which contains nucleotides 149 - 177 of the neomycin resistance gene (Genbank Accession number J01834)) and sites for EcoRI and XhoI, and primer BglII-linked 3' neomycin (SEQ. ID. NO: 14), which contains the reverse complement to the neomycin resistance gene nucleotides 922 - 945 and a BglII site. The plasmid pSV2Neo (ATCC 37149) was used as the template in the PCR reaction. The PCR fragment was cleaved with EcoRI and BglII and purified by gel electrophoresis and GeneCleanII. The two PCR fragments were directionally inserted in NotI and BglII digested

pPBM4-prime, giving rise to pPBM5 (Figure 1).

Analysis of neomycin phosphotransferase activity from pPBM5: COSM6 were transfected with pPBM5. 48 hr after transfection the cells were harvested and analyzed for the level of neomycin phosphotransferase activity expressed intracellularly using a NPTII ELISA kit #5307-543210 (Five-Prime To Three-Prime, Boulder, CO). The results of the assay are presented in Figure 4.

Construction of pPBM7: pPBM4 prime was used as the vector for subcloning the porcine DR α gene. Porcine MHC class II DR α gene (Hirsch et al., 1992, J. Immunol. 149, 841-846) was derived using SalI-linked 5' primer (SEQ. ID. NO: 15) and BglII-linked 3' primer (SEQ. ID. NO: 16) using pBSKSII-DR α as template plasmid. The BiP sequence was isolated by PCR from pL7gCAT using NotI-linked 5' primer (SEQ. ID. NO: 11) and SalI-linked 3' primer (SEQ. ID. NO: 17). The vector was generated by digesting pPBM4 -prime with NotI and BglII. The BiP IRES sequence and DR α sequence were then directionally subcloned in pPBM4 - prime, to give rise to pPBM7. pPBM7 contains the sequences for the mini-swine MHC class II cDNA genes for DR α and DR β .

Construction of pPBM8: pPBM7 was digested with BglII and the 5'-termini of the vector were dephosphorylated, using calf intestinal alkaline phosphatase (Promega Corp. Madison, WI). The BiP-Neomycin fragment to be inserted at the BglII site was isolated by PCR using BglII-linked 5' BiP primer (SEQ. ID. NO: 18) and BglII-linked 3' Neomycin primer (SEQ. ID. NO: 14) using pPBM5 as the template. To determine the orientation of the fragment in the sub-clones, DNA from the sub-clones was analyzed by digestion with a combination of several restriction enzymes. pPBM8 contains the sequences for the mini-swine MHC class II cDNA genes for DR α and DR β together with the G418 resistance gene.

Construction of pPBM9: After finishing the construction of pPBM8, it was discovered that the polylinker sequence had been eliminated at the time of constructing pPBM4-prime from pPBM4. Therefore, in order to insert the polylinker sequence into pPBM8, a 2.8 kb PCR fragment was generated using pPBM8 as template and the primers HindIII-linked 5' DR β (SEQ. ID. NO: 7) and ClaI-linked 3' SV40ori (SEQ. ID. NO: 5). The PCR fragment was cleaved with HindIII and ClaI and inserted between the ClaI and NotI sites of pPBM4, thereby creating pPBM9. pPBM9 contains the sequences for the mini-swine MHC class II cDNA genes for DR α and DR β .

Construction of pPBM10: A PCR fragment was generated using primers HindIII-linked 5' DR β (SEQ. ID. NO: 7) and ClaI-linked 3' Neomycin (SEQ. ID. NO: 19) and pPBM8 as the template. The PCR fragment was cleaved with HindIII and ClaI and purified by gel electrophoresis and GeneCleanII. The fragment was inserted into the vector fragment of NotI and ClaI digested pPBM4., thereby generating pPBM10. pPBM10 contains the sequences for the mini-swine MHC class II cDNA genes for DR α and DR β but differs from pPBM9 insofar as the SV40ori sequence was eliminated. pPBM10 was used for the generation of the retroviral packaging cell lines, designed to express the mini-swine MHC class II DR α / β heterodimer.

Expression of mini-swine MHC class II DR α / β from the polycistronic retroviral vectors. COSM6 cells were transiently transfected with (A) pPBM5 or (B) pPBM7. After 48 hr the cells were analyzed for the cell surface expression of the MHC class II DR α / β heterodimer. For detection of class II gene expression on the surface of the cells, the primary antibody used was ISCR3, which specifically recognizes an epitope of the DR α only when both DR α and DR β are presented as a heterodimer on the surface of the cell (Watanabe, M. et al., 1983, Transplantation 36: 712 -718). As a control for the staining procedure, the cells were also stained with the W632 antibody (Pescovitz, M.D. et al., 1984, J. Exp. Med. 160: 1495-

1505) that specifically recognizes monkey MHC Class I protein. The primary antibody reaction was followed by incubating the cells with the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin. The stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Figure 5 shows the data displayed in a histogram format as cell number (ordinate) vs. logarithm of fluorescence (abscissa). Dead cells were eliminated from the analysis by appropriate gating. In every instance, the data was matched and compared with the data obtained using a corresponding isotype control for that particular antibody. (76-2-11 for ISCR3 and 74-12-4 for W362). The superimposed histograms (Figure 5) show that 10-15% of the COSM6 cells transfected with pPBM7 which contains both the DR α and DR β cDNA genes in a polycistronic fashion manifested enhanced fluorescence intensity over cells transfected with pPBM5, which expresses only the DR β cDNA gene. The present data, therefore, emphasize that our polycistronic retroviral vectors are capable of expressing the heterodimeric DR α / β on the cell surface.

This demonstrates a system in accordance with the invention whereby expression of multiple genes in recombinant retroviral DNA-based constructs can readily be tested in transiently transfected COSM6 cells without doing a stable selection or without the need for packaging the recombinant retroviral DNA within its coat protein.

EXAMPLE 2

GENERATION OF RETROVIRAL PRODUCER CELL LINES EXPRESSING PORCINE MHC CLASS II DR α / β HETERODIMER USING pPBM8

This example reports the packaging of the constructs, capable of expressing three genes in a polycistronic fashion, within retroviral coat proteins and their ability to transduce cell line. A flow-diagram of the experiments reported in this Example is shown in Figure 6. High level expression was monitored from all three

genes from the G418 resistant clones, thereby conclusively demonstrating successful utilization of the BiP IRES sequence in a polycistronic fashion.

Transfection & Transduction of packaging cell lines:

The pPBMS retroviral vectors were used to transfect, using a transfection kit (Stratagene, CA), either the ecotropic packaging cell line, GP/E-86 or the amphotropic packaging cell line GP/E-envAM12., both packaging cell lines were obtained from Dr. Arthur Bank (Columbia University). Forty-eight hours after transfection, transiently expressed virus in the supernatant was recovered by filtration through a low protein binding syringe filter and added to a 20% to 30% confluent plate of recipient cells for transduction in presence of 8 mg/ml Polybrene (Sigma, St Louis, MO). GP/E-envAM12 cells were the recipient cells for the supernatant from GP/E-86 cells and vice versa. After 4 hours of infection, the viral supernatant was removed and complete medium was added to allow the cells to undergo 2-3 cycles of replication in 2-3 days. At this point the transduced cells were split in several dilutions and 0.8 mg/ml of active G418-containing medium was added. Under these growth conditions, only the cells expressing the neomycin phosphotransferase gene survived. After 14 days of selection in G418-containing medium, transduced sub-clones were picked and expanded, either as isolated sub-clones or mixtures of sub-clones. Meantime, the transfected cells were also grown in selective medium and subsequently analyzed for DR α / β expression.

Analysis of populations of cells transduced by recombinant retrovirus containing mini-swine MHC class II DR α /b cDNA genes:

Flow cytometry analyses: Flow cytometry analyses of the transduced cell lines were performed with antibodies such as ISCR3 (Watanabe et al. supra) or 40D (Pierres, M. et al., 1980, Eur. J. Immunol. 10: 950 -957), both of which recognize porcine MHC classII-DR α polypeptide only when it forms a heterodimer with the DR β

polypeptide. Figure 7 shows the data obtained from the mixture of sub-clones, referred to as "bulk". Neither of the untransfected cell lines exhibit enhanced fluorescence staining with the anti-class II antibody 40D (see Figure 7A and 7B). The mixture of sub-clones transfected with pPBM8 show a slight shift (see Figure 7C and 7D) while the transduced cells show a clear shift of fluorescence intensity (see Figure 7E and 7F). Indicative of the fact that the virus made by GP/E-envAM12 cells have transduced the GP/E-86 cells more efficiently so as to give rise to better expression of the DR α / β heterodimer is evident by the remarkable shift obtained by staining with 40D (Figure 7F) relative to that with the corresponding isotype. A statistical analysis indicated that 35% of the transduced G418 resistant GP/E-86 (denoted as AM>GP) cells were capable of expressing the DR α / β heterodimer on the surface.

Analysis of individual sub-clones generated by transfection of packaging cell lines using pPBM8:

After analyzing the bulk population of transfected and transduced cells by flow cytometry, the bulk population was scored for individual G418 resistant sub-clones by limiting dilution. Individual sub-clones were subsequently analyzed by flow cytometry. The representative fluorescence histograms for an ecotropic as well as an amphotropic producer subclone are shown in Figure 8. Figure 8A shows the superimposed histograms of the control (untransfected, G418 sensitive) GP/E-86 cells, obtained by staining with surface antibody 40D for porcine DR α / β heterodimer and with the isotype control antibody 76-2-11. The histogram obtained by staining cells with the antibody B1094 (Dr. D. H. Sachs, Massachusetts General Hospital, Boston, MA), specific for mouse MHC Class I antigen served as a positive control for successful staining. Absence of any shift of peak of the fluorescence intensity in the 40D-histogram with respect to that of the isotype histogram indicates that the untransfected packaging cell line does not express the MHC

class II DR α / β heterodimer (Figure 8A). However, as shown in Figure 8B, the transfected cell line GP12-2 gives rise to a histogram which shows a clear shift of a fluorescence peak with the 40D antibody. The untransfected cell line GP/E-envAM12 does not express the MHC class II DR α / β heterodimer (Figure 8C), while the transfected cell line AM1¹, -6 shows a high level of expression of the DR α / β heterodimer (Figure 8D). Statistical analyses as obtained by legitimate gating of the fluorescence histograms showed that 42% and 65% of the total cell population express DR α / β heterodimer from the ecotropic and amphotropic producer sub-clones respectively.

Analysis of the integrated DNA

Using cellular DNA as the template, polymerase chain reactions were performed using primers HindIII-linked 5'DR β (SEQ. ID. NO: 7) and BgIII-linked 3'DR α (SEQ. ID. NO: 16) to analyze the integrated DNA fragments present in the chromosomes of the individual producer sub-clones. PCR fragments were sequenced in order to ascertain the integrity of the DNA, i.e., to ensure that the recombinant retroviral vector has integrated in the cellular chromosome without undergoing any gene rearrangement within the vector sequence.

Appearance of an 1.8 kb PCR fragment was as expected from the theoretical estimation (Figure 9). The sequence analysis revealed that the integrated genome did not undergo any rearrangement during the first week of cellular expansion. The generation of a shorter band which appeared even in the negative control was either an artifact or had been generated from the non-specific annealing of the primers at some cellular sequences.

Viral titer

The titer of virus stocks produced from both the producer cell lines on NIH-3T3 cells were determined to be about 2×10^5 .

Thus, these data strongly support the effectiveness of using

a polycistronic vector in expressing multimeric protein for the purpose of retroviral gene therapy.

EXAMPLE 3

ANALYSIS OF DNA FROM CELLS TRANSFECTED WITH pPBM8

During continued passage of the producer cell lines obtained after transfecting with pPBM8, it was noted that the cells gradually lost the ability to express the mini-swine MHC class II DR α / β heterodimer. Southern blot analysis of the integrated DNA was performed. Genomic DNA was isolated from the cell lines by rinsing a 100 % confluent culture plate twice with 1X phosphate buffered saline (PBS). The cell were lysed by the addition of 400 μ l 2X lysis buffer (2X lysis buffer contains 0.2 M Tris-HCl, pH 7.0, 0.1 M Na₂EDTA, 2% SDS, 0.1 M NaCl, 400 μ g/ml proteinase K. The lysed cells were scraped at room temperature and incubated overnight at 55°C. The solution was then extracted five times with buffer-saturated phenol-chloroform. The DNA was ethanol-precipitated and re-dissolved in 10 mM Tris-HCl, pH 7.0, 1 mM Na₂EDTA, 50 μ g/ml DNase-free RNase at 37°C for 1 hour. The DNA was re-extracted with phenol-chloroform and ethanol-precipitated. The DNA was re-suspended in 10 mM Tris-HCl, pH 7.0, 1 mM Na₂EDTA. For Southern blot analysis the DNA (20 μ g) was digested with SacI which cleaves the retroviral vectors only in the 5' and 3' LTRs.. The digested DNA was electrophoresed through 1% agarose and transferred to a nitrocellulose filter (Scheicher and Schuell, Keene, NH). The membrane was hybridized overnight under the appropriate conditions for a randomly primed radioactive (using the method described in the Boehringer-Mannheim random priming kit) probe (either the DR α or the DR β fragment). The membranes were washed under stringent conditions and exposed to Kodak X-OMAT film. Figure 10A shows an autoradiograph of SacI digested DNA from the cell lines probed for the presence of sequences hybridizing to the pig DR β probe. The multiplicity of bands corresponding to 4.9 kb, 3.6 kb and 3.0 kb can be interpreted using the schematic diagram in Figure 10B and

correspond to the sizes expected if the integrated DNAs had undergone homologous recombination between the copies of the BiP IRES sequences. The frequency of the deletion events in the transfected retroviral packaging cell lines can be explained by the ability of the produced virus to super-infect the host cell and subsequent retrovirus-mediated homologous recombination. The phenomenon of super-infection has been reported (Muenchau et. al., 1990, Virology 176: 262-265). There are several recent reports in the literature regarding the occurrence of retrovirus-mediated homologous recombination (Zhang and Temin, 1993, Science, 259: 234-238; Temin, H., 1993, Proc. Natl. Acad. Sci. USA, 90: 6900-6903). The presence of bands corresponding to sizes that cannot be readily explained may be the result of recombination events between short stretches of non-obvious sequence homology. It is of interest to note that the intensity of the band corresponding to the full-length proviral DNA sequence (the 4.8 kb band) correlates, for different sub-clones, with the level of expression of the DR α / β heterodimer, (Figure 8)

EXAMPLE 4

GENERATION AND ANALYSIS OF PACKAGING CELL LINES RETROVIRALLY TRANSDUCED WITH pPBM14

Construction of pPBM13

pPBM7 (Figure 1) was modified so that the DR β cDNA gene could be under the direct control of the 5'LTR for translational initiation. In order to do this, the EcoRI-BspEI fragment which contains the 5' cDNA sequence of DR β from pBSKSII-DR β plasmid (Gustaffson, K. et al., 1990, Proc. Natl. Acad. Sci. USA, 87: 9798-9802) was used to replace the similar fragment from pPBM7 which contained not only the 5' sequence of DR β but also the first BiP IRES sequence. The new construct, pPBM13, (Figure 11), thus contains the DR β gene under the translational control of the 5'LTR. The DR α cDNA gene remains under the translational control of the

BiP IRES. pPBM13 does not contain sequences for selection in eukaryotic cells.

Construction of pPBM14

In order to incorporate a drug resistance marker, pPBM13 was modified as follows. A DNA fragment containing a second, non-homologous IRES, was generated using the polymerase chain reaction - the plasmid pGlEN (Genetic Therapy Inc. Gaithersburg, MD) was subjected to the polymerase chain reaction in the presence of primers BglII-XhoI-linked 5' EMC (SEQ. ID. NO: 20) and BglII-linked 3' neomycin (SEQ. ID. NO: 14). The fragment thus amplified contains the 5' untranslated region of the encephalomyocarditis virus (EMCV) and the neomycin phosphotransferase gene. The DNA fragment was cleaved with BglII and gel purified before being inserted into the BglII site in pPBM13, thereby generating pPBM14 (Figure 11).

Analysis of MHC class II DRa/b expression following transfection of pPBM13 into COSM6 cells:

Transfection into COSM6 cells was performed, using CsCl purified supercoiled plasmid DNA, pPBM13, by the DEAE dextran sulfate method as described in Example 1. 48 hr following transfection, the cells were harvested by scraping the culture plates in the presence of 1X phosphate-buffered saline (PBS - 1X PBS is 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3) containing 0.2% Na₂EDTA. The collected cells were rinsed twice with 1X PBS and 10⁶ cells (in 100 µL) were added to each well of a 96 well micro-titer plate. Appropriately diluted primary antibodies were added to the cells which were stained for 30 min. at room temperature. The non-reactive antibodies were washed off and FITC-conjugated secondary antibody was added for 30 min. at 4°C. After stringent washing of the cells, the cells were analyzed by flow cytometry. Phosphor-iodide gating (Becton-Dickinson Manual) was used to gate out dead cells. Figure 12 shows the

results of the flow cytometry analysis, the data is represented in histogram format using the number of cells as the ordinate and the fluorescence intensity as the abscissa. It is apparent from Figure 12 that the vector pPBM13 expresses the mini-swine MHC class II DR α / β heterodimer as efficiently as did pPBM7.

Transfection, using pPBM14, into GP/E-86 and subsequent transduction into the PA317 retroviral packaging cell line.

The ecotropic GP/E-86 retroviral packaging cell line was transfected with pPBM14 using the Mammalian Transfection Kit (Stratagene, La Jolla, CA). The transiently expressed virus from GP/E-86 cells was harvested 48 hr after transfection, filtered through a low protein binding 0.45 μ M filter (Gelman Sciences, Ann Arbor, MI) and was used to transduce the amphotropic packaging cell line PA317 (ATCC CRL 9078). After transducing the cells for 18 hr with a retroviral titer of 104/ml, the transduced cells were split in appropriate dilutions and 500 μ g/ml active G418 was added to the medium in order to select G418 resistant cells. To monitor the transfection efficiency, the transfected GP/E-86 cells were plated in presence of 1 mg/ml active G418 containing media in limited dilutions for scoring the G418 resistant clones.

Analysis of MHC class II DR α / β expression following transfection of pPBM14 into (a) GP/E-86 cells and (b) subsequent transduction of PA317 cells

The results of flow cytometry analysis of GP/E-86 cells stably transfected with pPBM14 are shown in Figure 13. All of the G418 resistant cells expressed the MHC class II DR α / β heterodimer, to differing extents (see Figure 13 for the flow cytometry analysis of the four individual transfected G418 resistant sub-clones).

Transiently expressed ecotropic retrovirus harvested 48 hrs after transfection into GP/E-86 cells was used to transduce the amphotropic cell line PA317. Transduced sub-clones were obtained

following selection in G418. Flow cytometry analysis of the G418 resistant clones revealed that more than 90% of the individual transduced sub-clones were positive for expression of the MHC class II DR α / β heterodimer. Figure 14A shows the negative control, i.e. PA317 cells transduced with supernatant generated from mock-transfected GP/E-86 cells. The majority of the histograms obtained with antibody 40D showed a single clear shift of peak fluorescence intensity (five representative histograms are shown in Figure 14B-F). One histogram (Figure 14F) showed a double shift of peak fluorescence intensity, probably indicating a mixed population of MHC class II DR α / β heterodimer expressing sub-clones.

Figure 15 displays a bar graph showing the percentage of transduced cells in a particular population which demonstrate a higher level of fluorescence intensity with the antibody recognizing the DR α / β heterodimer relative to that recognizing the isotype control.

In order to determine whether the producer clones lose their ability to express the MHC class II DR α / β heterodimer, several clones were expanded in the presence of sub-optimal concentrations of G418 (200 μ g/ml for the PA317-derived lines and 500 μ g/ml for the GP/E-86 derived lines. After approximately 30 cell divisions the producer clones were re-analyzed by flow cytometry analysis. The expression levels remained constant, indicating that the integrated retroviral sequences were not undergoing the rapid rearrangement/deletion phenomenon that had been observed with the lines derived from pPBM8 (data not shown).

Analysis of viral RNA

In order to ascertain that the expression of the MHC class II DR α / β heterodimer was the consequence of expression of sequences present in a single proviral integration event, RNA was extracted from pelleted virus and an RNA slot blot analysis was performed.

The autoradiograph in Figure 16 indicates that there are equivalent amounts of DR α and DR β RNA for the clones as discerned by the intensity of hybridization with the respective cDNAs as radioactive probes. This argues in favor of the integrity of the proviral genome in the producer sub-clones. Had recombination/deletion occurred we would have expected a difference in the proportionality of the RNA transcripts specific for either DR α or DR β because it is likely that viruses bearing two different types of RNA genomes would express different amounts of RNA.

Assay for viral titer.

Virus was harvested from virus producing cells by adding 5 ml of fresh culture media on day 1 to 80% confluent 10 cm dishes for 16 hr. On day 2, the supernatant containing the virus was filtered through 0.45 μ M low protein binding filter and diluted appropriately with media. Then, 0.5 ml of the supernatant from various dilutions was added to 40% confluent dishes of recipient NIH-3T3 (ATCC CRL 1658) cells in presence of 4 μ g/ml of Polybrene. On day 3, fresh media containing 1 mg/ml active G418 was added to the transduced cells and neomycin resistant colonies were scored after 10-12 days by fixing the cells in methanol and staining the cells with Giemsa stain. Virus titer was calculated in colony forming units/ml (cfu/ml) as shown in Table 1.

Assay for presence of replication competent retrovirus by amplification of transduced NIH/3T3 cells.

The stable G418 resistant NIH-3T3 cells were amplified and tested for presence of replication competent retrovirus (RCR). If any contamination or recombinatorial event was able to give rise to RCR in the amphotropic viral supernatant that was used to infect NIH-3T3 cells, expansion of the NIH-3T3 cells would result in a several-fold amplification of the RCR. By this amplification technique, breakout of a single RCR could be detected by doing marker rescue S+L- assay using PG4 as an indicator cell line

(Haapala, D. et al., 1985, J. Virol. 53: 827-833)or by PCR using primer sequences corresponding to the sequences of envelope protein. Details of these methods are as described (Anderson, W.F., 1993, Human Gene Therapy, 4: 31-321). The inability of the supernatants from the respective NIH-3T3 cells to form foci on the PG4 cells indicated that replication competent virus was not present or, had not been generated in this particular combinations of retroviral vectors and packaging cell lines.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT(S): Banerjee, Papia T.
LeGuern, Christian A.
Seed, Brian

(ii) TITLE OF INVENTION: Polycistronic Retroviral Vector Containing Multiple
Related Translational Initiation Capability

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Carella, Byrne, Bain,
Gilfillan, Cecchi, Stewart &
Olstein
(B) STREET: 6 Becker Farm Road
(C) CITY: Roseland
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch diskette
(B) COMPUTER: IBM XT
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(vi) PARENT APPLICATION DATA

(A) APPLICATION NUMBER: 08/006,478
(B) FILING DATE: January 20, 1993
(C) CLASSIFICATION: U.S. Preliminary Class 435

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Herron, Charles J.
(B) REGISTRATION NUMBER: 28,019
(C) REFERENCE/DOCKET NUMBER: 61750-95

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-994-1700
(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 17 BASES

SUBSTITUTE SHEET (RULE 26)

-32-

- (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:
GCTCTTCGGA CCCTGCA 17
- (3) INFORMATION FOR SEQ ID NO: 2
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 17 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: YES
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:
CGAGTGAGGG GTTGTGG 17
- (4) INFORMATION FOR SEQ ID NO: 3
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 17 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:
CGATTAGTCC AATTTGT 17
- (5) INFORMATION FOR SEQ ID NO: 4
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 17 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: YES
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:
TCTATCTATG GCTCGTA 17

-33-

- (6) INFORMATION FOR SEQ ID NO: 5
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 27 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: YES
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:
GTCAATCGAT AGCTTTTTC AAAAGCC 27
- (7) INFORMATION FOR SEQ ID NO: 6
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 33 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6:
GCGAATCGAT AGATCTACTC CGCCCATCCC GCC
- (8) INFORMATION FOR SEQ ID NO: 7
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 30 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 7:
GGCTCAAGCT TCAGCATGGC ATGTCTGTGT 30
- (9) INFORMATION FOR SEQ ID NO: 8
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 45 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: YES

-34-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 8:
AAGGAAAAAA GCGGCCGCTT ATCAGGAGGC CTGTTGGCTG AAGGG 45

(10) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 25 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 9:
AATTGGCCAA TATTTTGGAC GCGTC 25

(11) INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 24 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 10:
ATTGACGCGT CCAAATATT GGCC 24

(12) INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 44 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 11:
AAGGAAAAAA GCGGCCGCCG CGACGCCGGC CAAGACAGCA CAGA 44

(13) INFORMATION FOR SEQ ID NO: 12

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 28 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE

-35-

- (D) TOPOLOGY: LINEAR
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 12:
GATCGAATTC AGCCAGTTGG GCAGCAGC 28
- (14) INFORMATION FOR SEQ ID NO: 13
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 47 BASES
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 13:
TCGAATTCCA AGGCTCGAGC ATGATTGAAC AAGATGGATT GCACGCA 47
- (15) INFORMATION FOR SEQ ID NO: 14
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 36 BASES
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 14:
CACAAGATCT TATCAGAAGA ACTCGTCAAG AAGGCG 36
- (16) INFORMATION FOR SEQ ID NO: 15
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 33 BASES
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 15:
TTCCGGTCGA CGCCGCCAAA ATGACCATAC TTG 33
- (17) INFORMATION FOR SEQ ID NO: 16
- (i) SEQUENCE CHARACTERISTICS

-36-

- (A) LENGTH: 36 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 16:
GGACAGATCT TATCACAGAG GCCCTCGGTG TTCAGT 36
- (18) INFORMATION FOR SEQ ID NO: 17
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 36 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 17:
GATCAGCCGG TCGACAGCCA GTTGGGCAGC AGCAAG 36
- (19) INFORMATION FOR SEQ ID NO: 18
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 33 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 18:
GGAATCAGAT CTCGACGCCG GCCAAGACAG CAC 33
- (20) INFORMATION FOR SEQ ID NO: 19
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 33 BASES
(B) TYPE: NUCLEIC ACID
(C) STRANDEDNESS: SINGLE
(D) TOPOLOGY: LINEAR
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 19:
CCATCGATGG TAATCAGAAG AACTCGTCAA GAA 33

-37-

- (21) INFORMATION FOR SEQ ID NO: 20
- (i) SEQUENCE CHARACTERISTICS
 - (A) LENGTH: 32 BASES
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: SINGLE
 - (D) TOPOLOGY: LINEAR
 - (iii) HYPOTHETICAL: NO
 - (iv) ANTISENSE: NO
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 20:
CAGGAAGATC TCTCGAGGAT CAATTCGCC CC 32

-38-

TABLE 1

<u>SUBCLONE</u>	<u>TITER(CFU/ML)</u>
GPA 25	3×10^4
GPA 26	1×10^3
GPA 38	6×10^3
GPA 59	5×10^4
GPA 62	1×10^6
GPA 72	1×10^6
GPA 75	2×10^5
GPA 77	1×10^6
GPA 80	4×10^4
GPA 141	1×10^5

SUBSTITUTE SHEET (RULE 26)

WHAT IS CLAIMED IS:

1. A retroviral vector comprising
 - (i) a single transcription regulatory sequence at the 5' region thereof;
 - (ii) a first DNA coding sequence;
 - (iii) at least one additional DNA coding sequence;
 - (iv) an IRES controlling the translation of each DNA coding sequence of (iii), wherein the vector expresses multiple independent polypeptides capable of posttranslational combination to form at least one functional multimeric protein.
2. The retroviral vector of claim 1 wherein the IRES is a BiP IRES.
3. The retroviral vector of claim 2 which expresses multiple independent polypeptides capable of posttranslational combination to form at least one functional multimeric protein.
4. The retroviral vector of claim 2 which produces a single, functional heterodimeric protein of the major histocompatibility complex.
5. The retroviral vector of claim 1 which is selected from the group of pL7gCAT, pPBMI through pPBM14 and derivatives of any of them.
6. A cell containing the retroviral vector of claim 1.
7. The cell of claim 6 which is mammalian.
8. The cell of claim 7 which is human.

9. A retroviral vector comprising
- (i) a single transcription regulatory sequence at the 5' region thereof;
 - (ii) a first DNA coding sequence;
 - (iii) at least two additional DNA coding sequences; and
 - (iv) IRESs controlling the translation of each such additional coding sequence to express at least one functional multimeric protein, wherein at least one of such IRESs is a cellular IRES.
10. The retroviral vector of claim 9 wherein the cellular IRES is a mammalian IRES.
11. The retroviral vector of claim 10 wherein the cellular IRES is a BiP IRES.
12. The retroviral vector of claim 9 wherein at least one IRES of (iv) is a viral IRES.
13. A method for inducing immune tolerance in a human recipient for graft transplantation which comprises re-introducing, into said human recipient, cells explanted from said human and made to contain a vector that expresses multiple independent mammalian polypeptides that posttranslationally combine to produce a functional, multimeric protein.
14. The method of claim 13 wherein said DNA coding sequences code for polypeptides of a functional heterodimeric protein of the major histocompatibility complex of a non-human.
15. The method of claim 14 wherein the non-human is porcine.
16. The method of claim 14 wherein the non-human is a primate.

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/00650

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : 435/69.1, 320.1, 240.2; 424/93A, 93T

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 240.2; 424/93A, 93T

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medicine, Medline, Biotech

Search Terms: retroviral vector, IRES, heterodimeric protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Vol. 353, issued 05 September 1991, D. G. Macejak et al., "Internal Initiation of Translation Mediated by the 5' Leader of a Cellular mRNA", pages 90-94, see page 93.	1-16
Y	Journal of Virology, Vol. 65, No. 9, issued September 1991, M. A. Adam et al., "Internal Initiation of Translation in Retroviral Vectors Carrying Picornavirus 5' Nontranslated Regions", pages 4985-4990, see Figure 1.	1-16

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

14 APRIL 1994

Date of mailing of the international search report

25 APR 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

David Guzo

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

In. International application No.

PCT/94/00650

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Vol. 20, No. 6, issued 1992, R.A. Morgan et al., "Retroviral Vectors Containing Polycistronic Internal Ribosome Entry Sites: Development of a Putative Gene Transfer System and Applications to Human Gene Therapy", pages 1293-1299, see Figures 5-6.	1-16
Y	Journal of Virology, Vol. 63, No. 4, issued April 1989, S. K. Jang et al. , "Initiation of Protein Synthesis by Internal Entry of Ribosomes into the 5' Nontranslated Region of Encephalomyocarditis Virus RNA In Vivo", pages 1651-1660, see Figure 1.	1-16

INTERNATIONAL SEARCH REPORT

International application No.

US94/00650

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A01N 63/00; A61K 48/00, 49/00; C12P 21/06; C12N 5/00, 5/16, 5/22, 15/00, 7/01

THIS PAGE BLANK (USPTO)

FIG. 1A

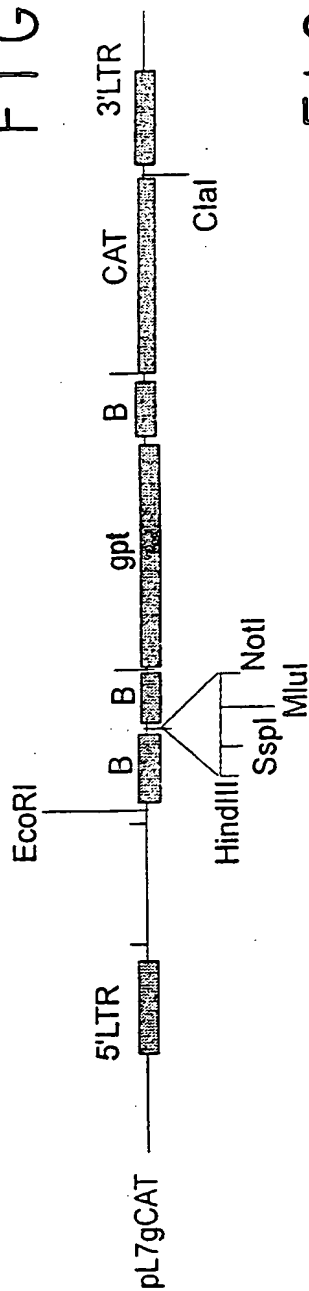


FIG. 1B

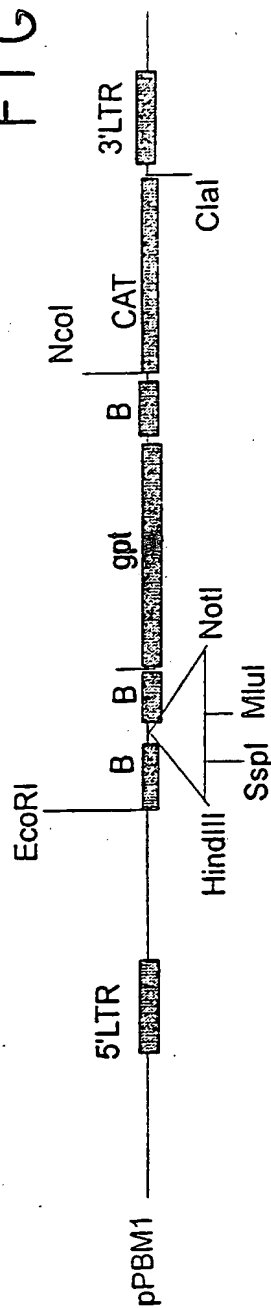


FIG. 1C

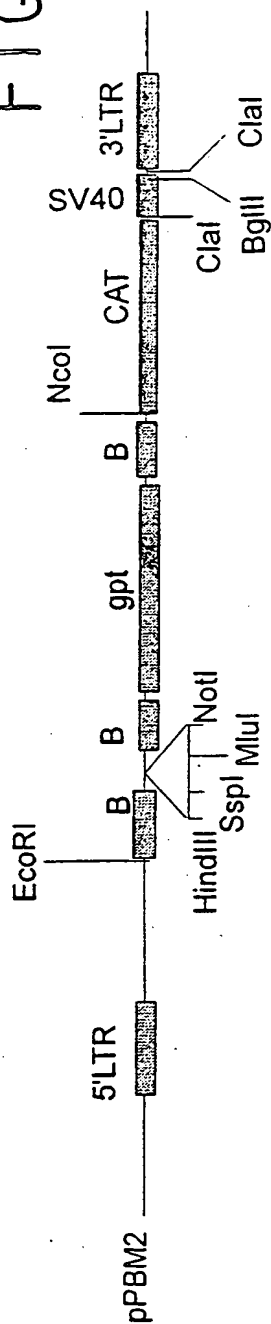
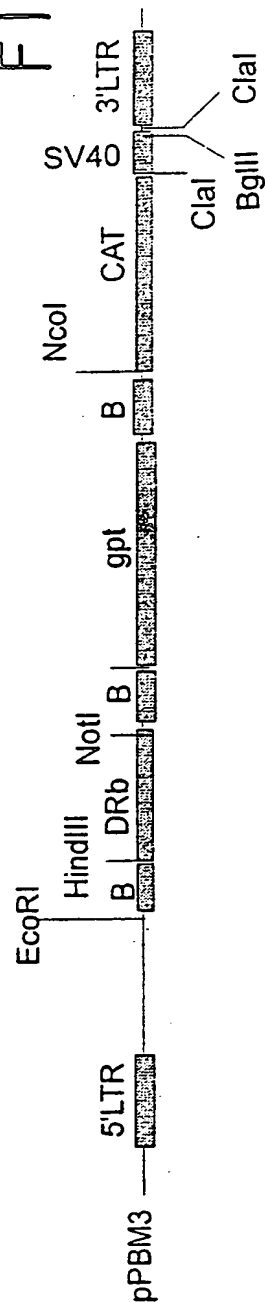


FIG. 1D



THIS PAGE BLANK (USPTO)

FIG. 1E

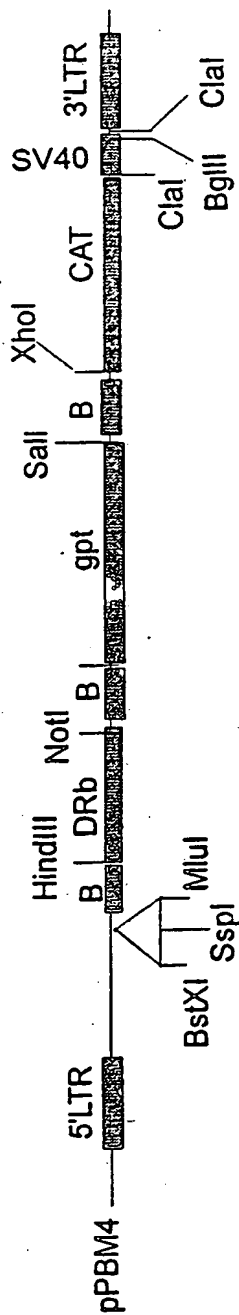


FIG. 1F

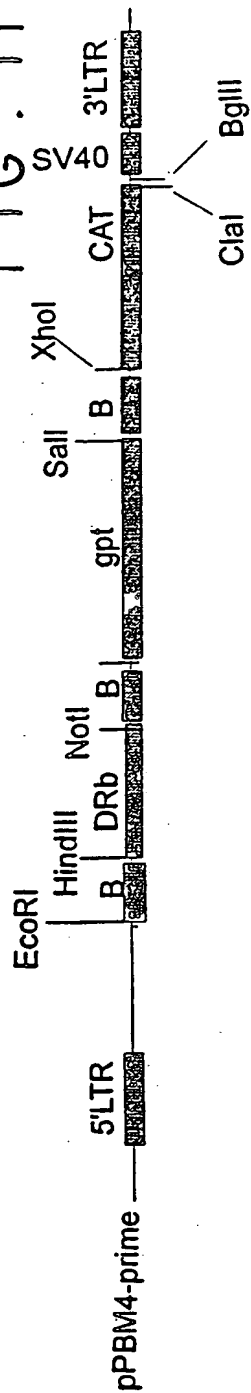


FIG. 1G

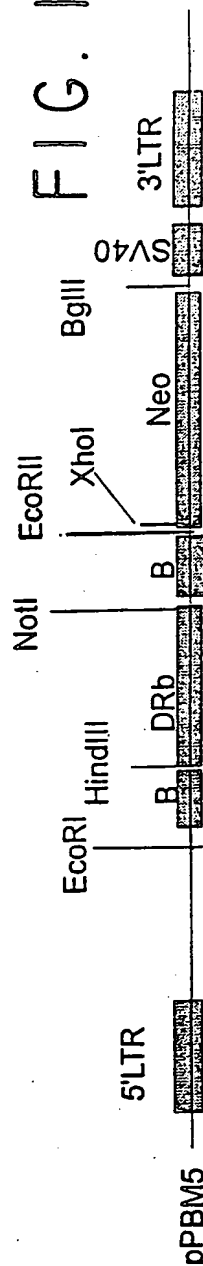
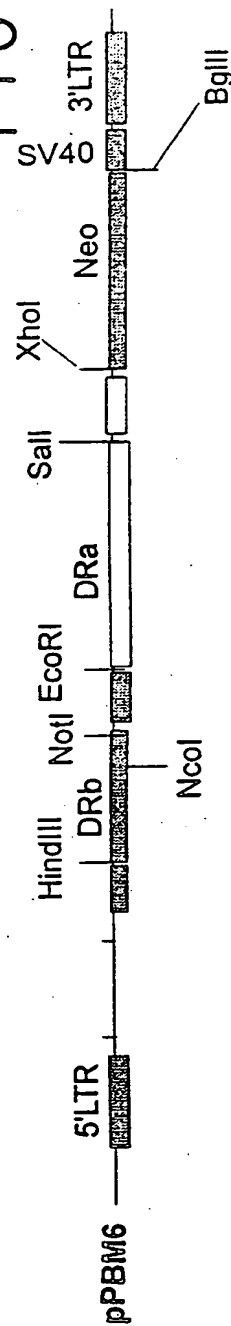


FIG. 1H



THIS PAGE BLANK (USPTO)

FIG. II

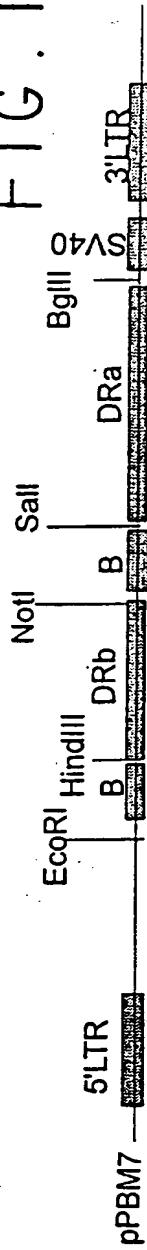


FIG. IJ

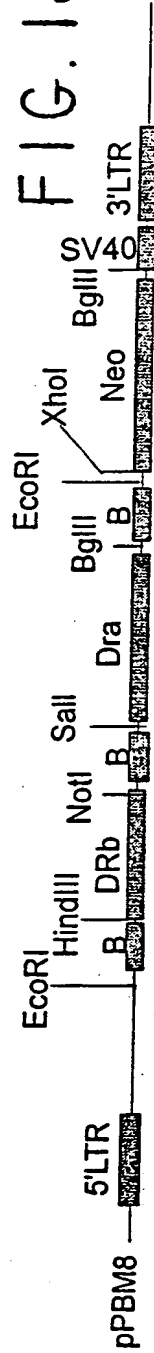


FIG. IK

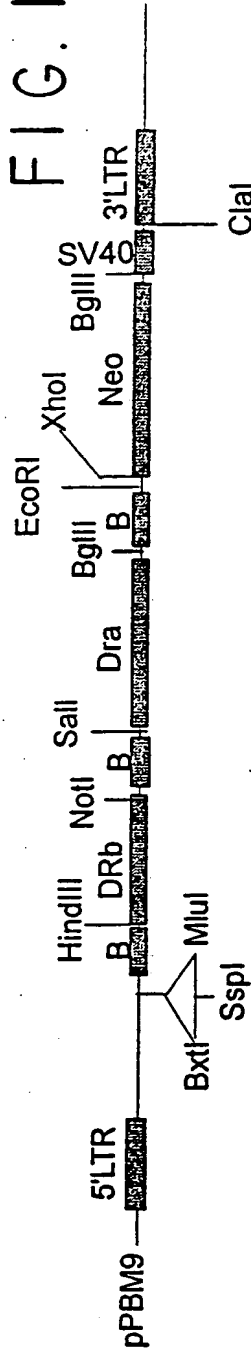
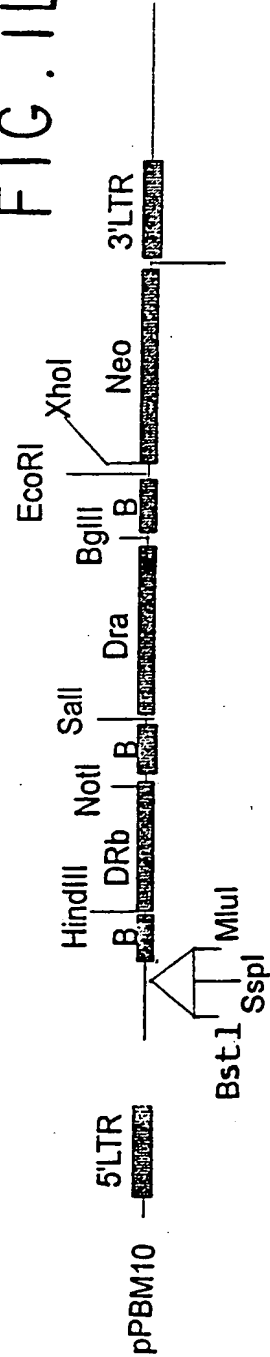


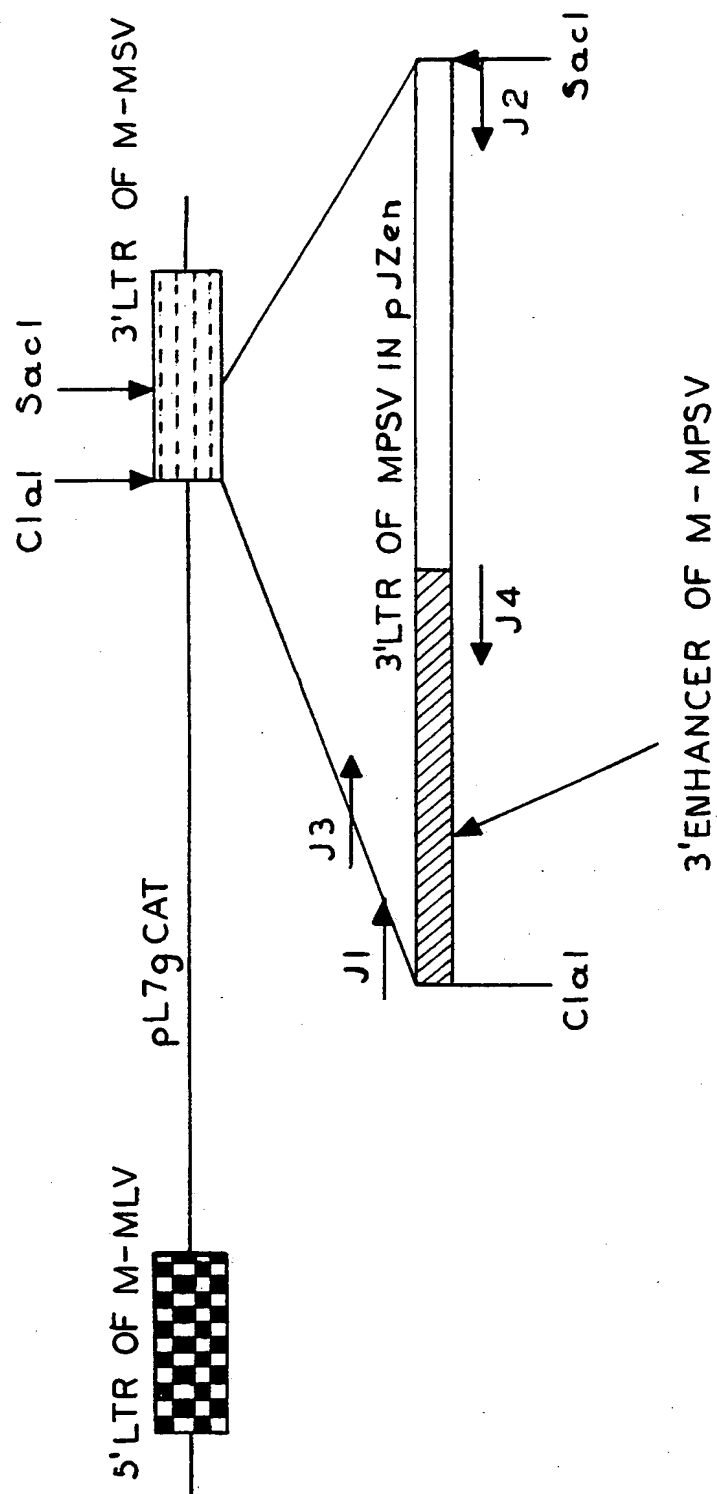
FIG. IL



THIS PAGE BLANK (USPTO)

4/18

FIG. 2

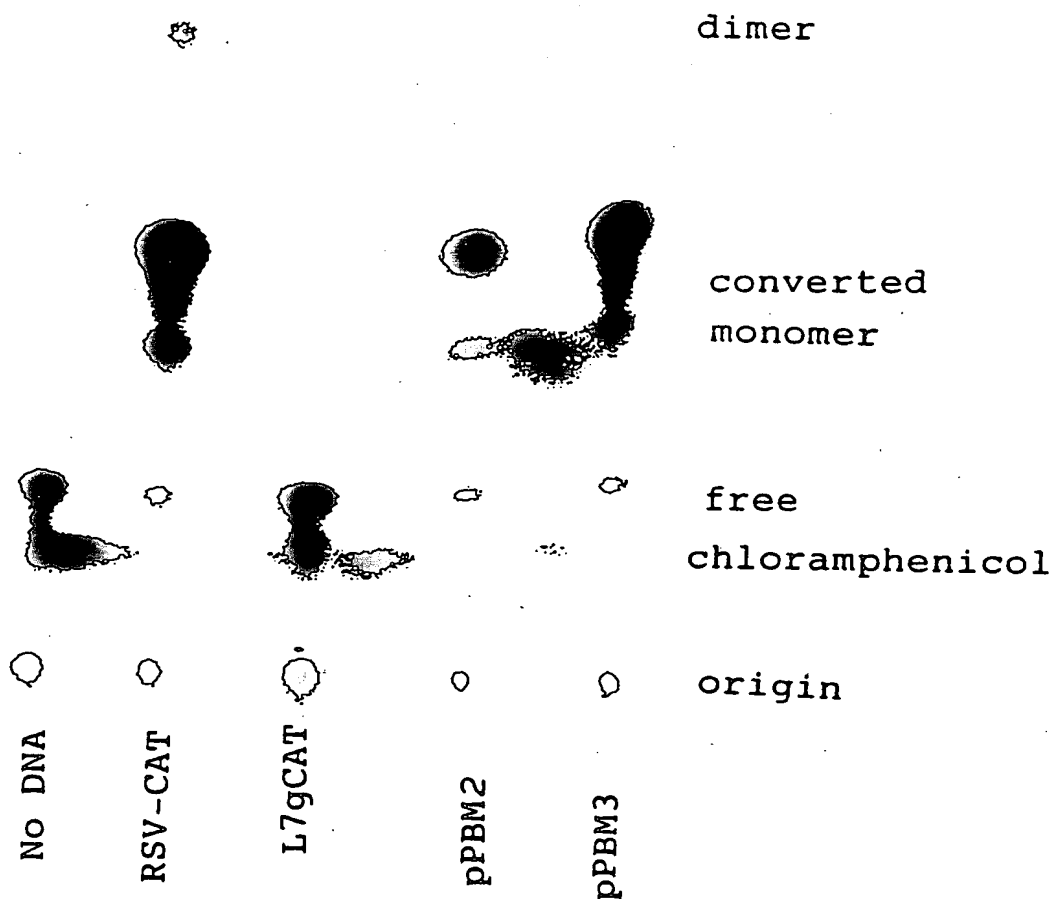


SUBSTITUTE SHEET (RULE 26)

THIS PAGE BLANK (USPTO)

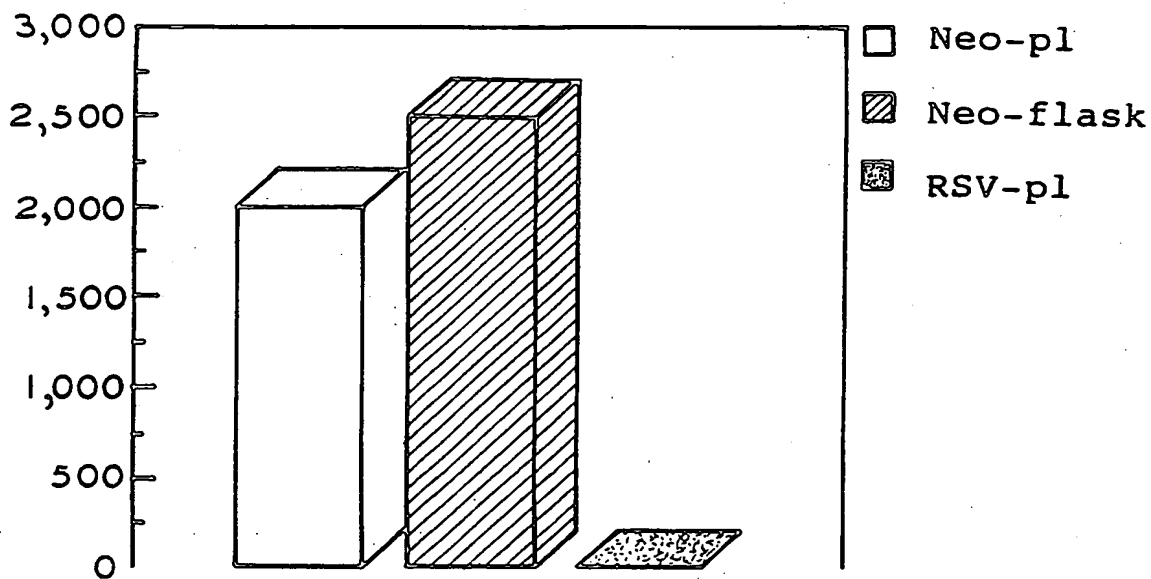
5/18

FIG. 3



Neomycin pg/ml

FIG. 4



THIS PAGE IS BLANK

6/18

FIG. 5A

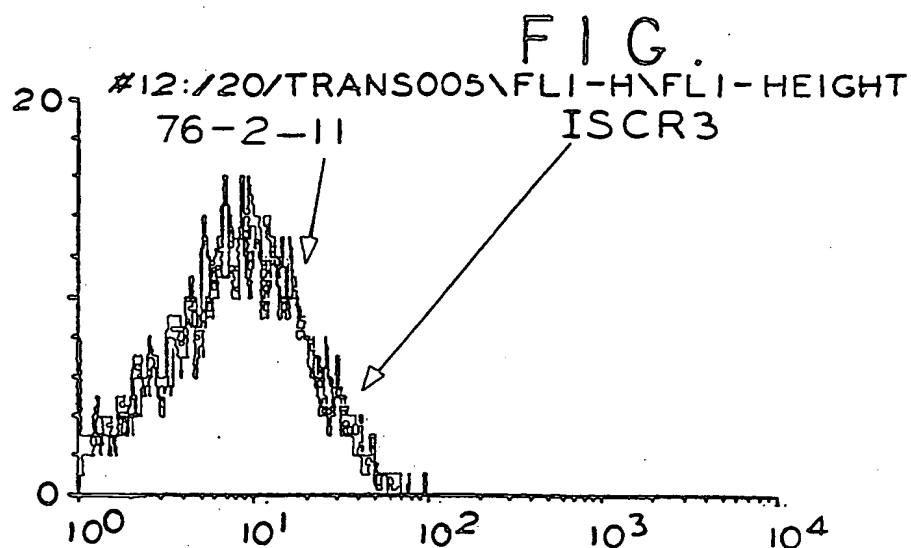
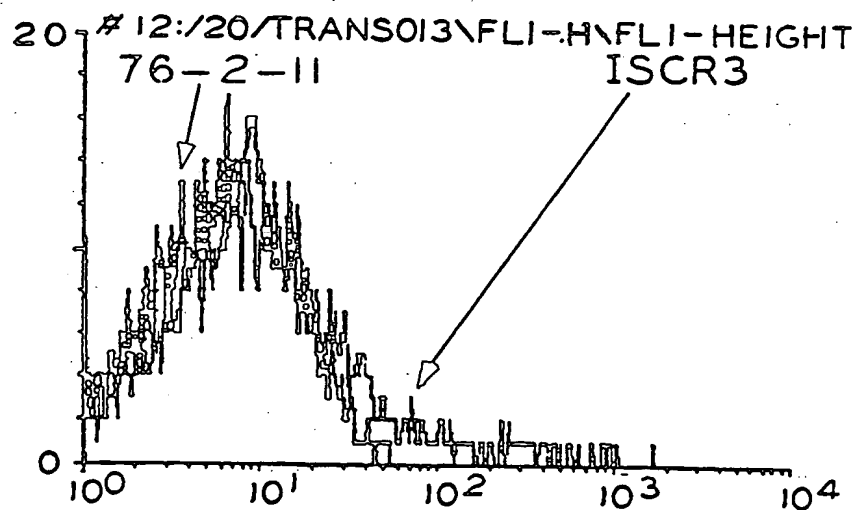


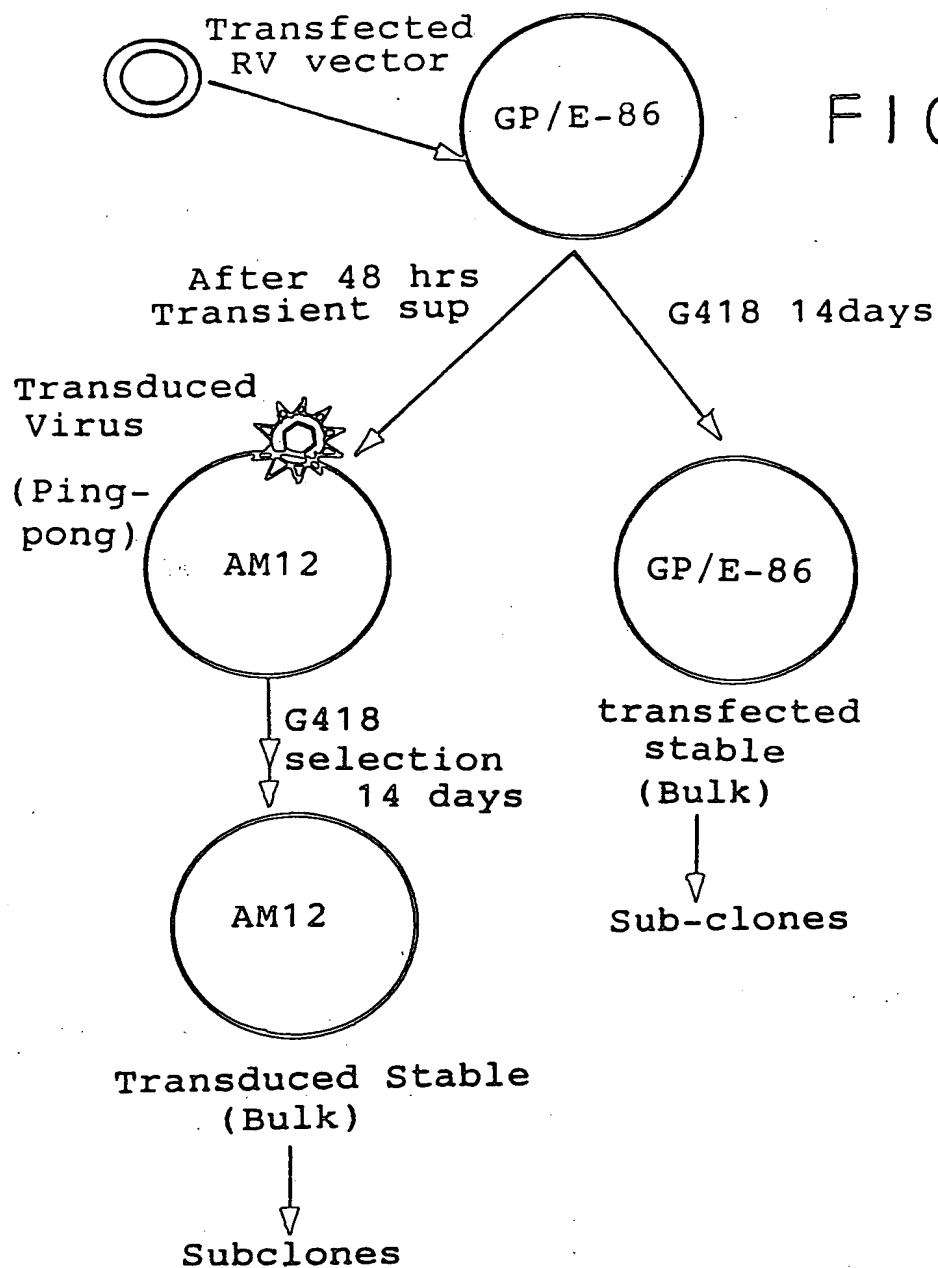
FIG. 5B



SUBSTITUTE SHEET (RULE 26)

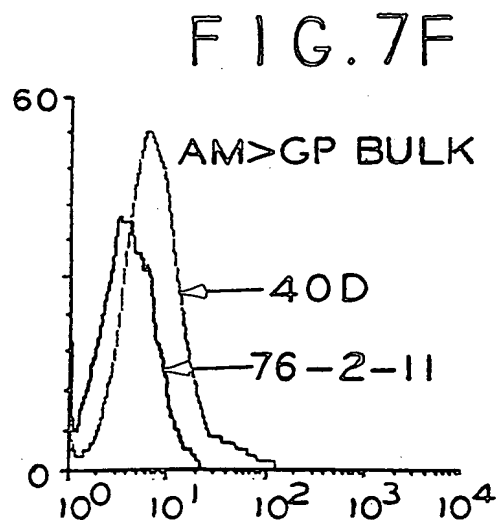
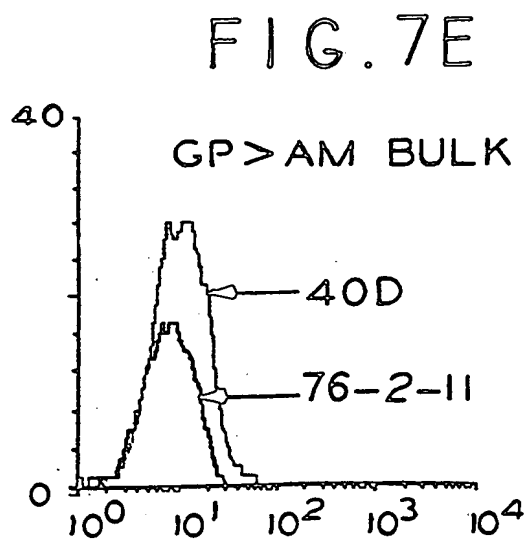
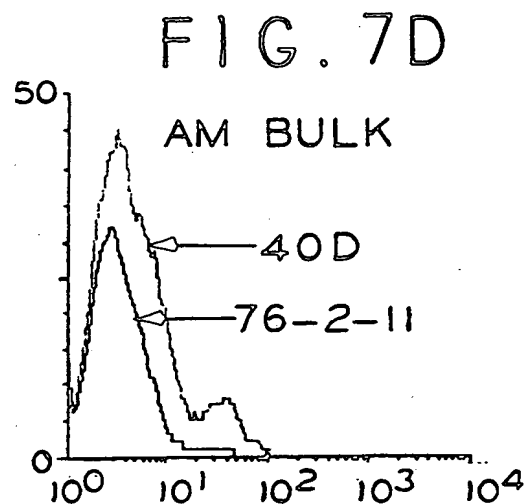
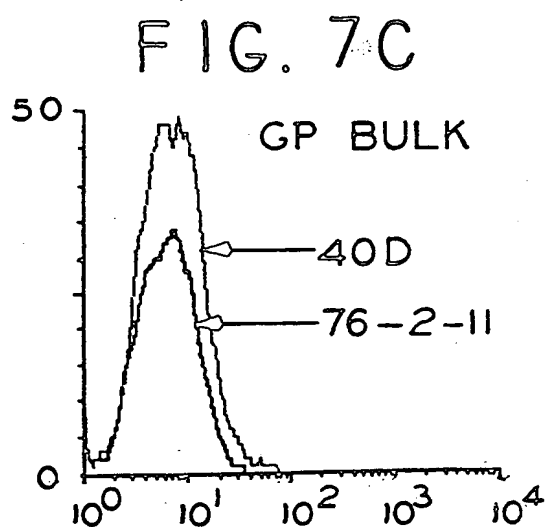
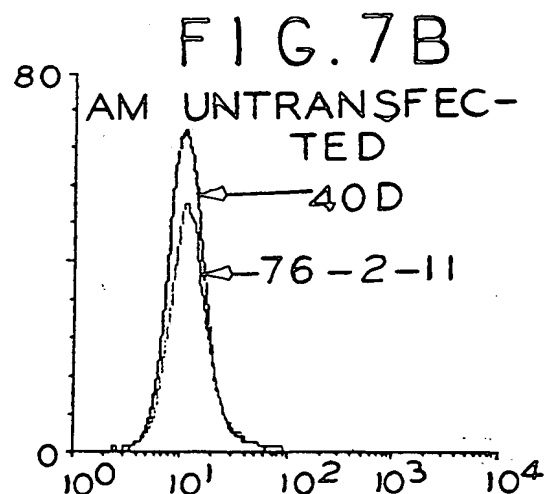
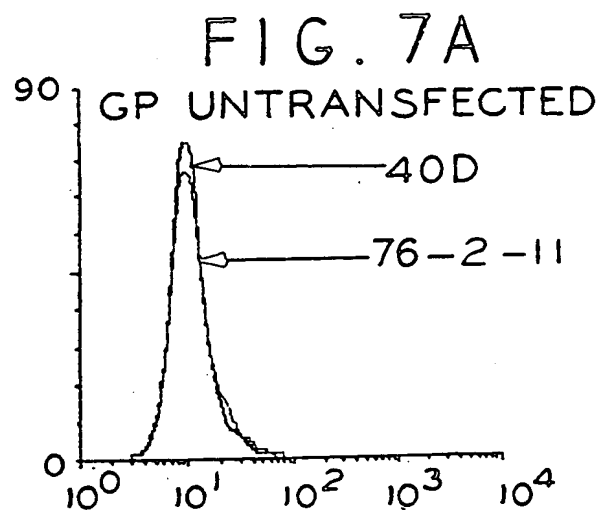
THIS PAGE

7/18



THIS PAGE BLANK (USPTO;

8/18



THIS PAGE BLANK (USPTO)

9/18

FIG. 8A

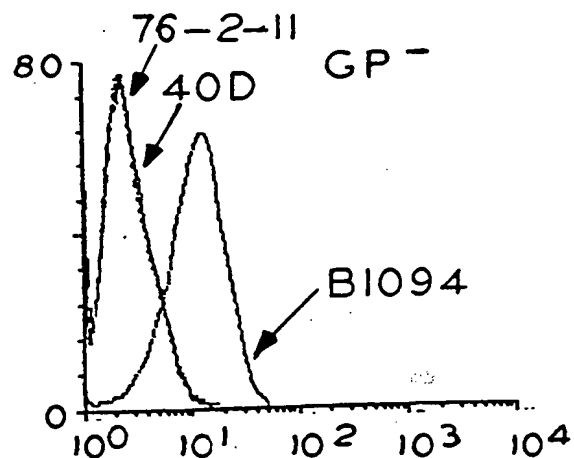


FIG. 8B

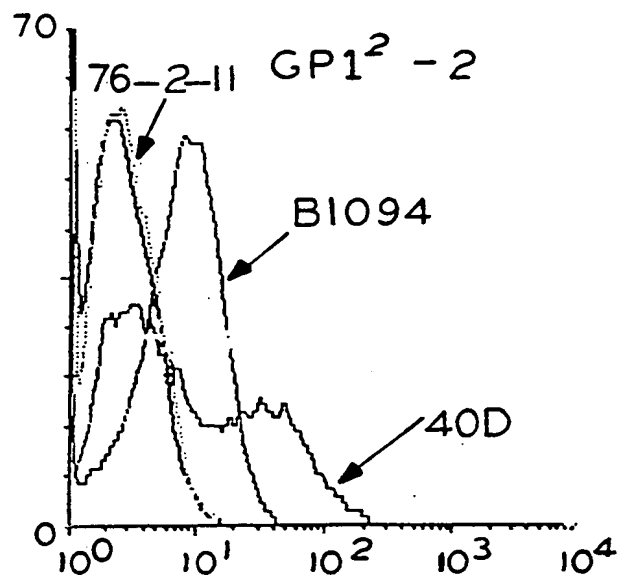


FIG. 8C

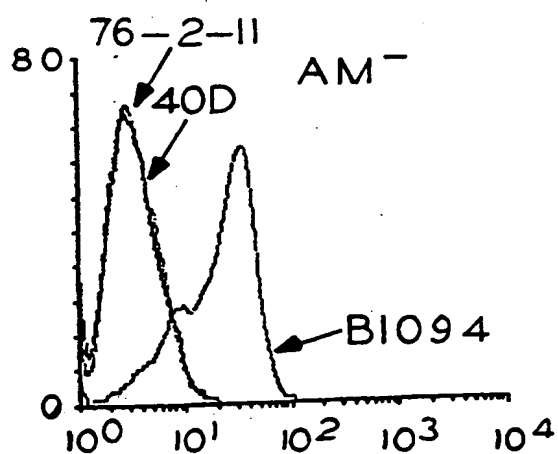
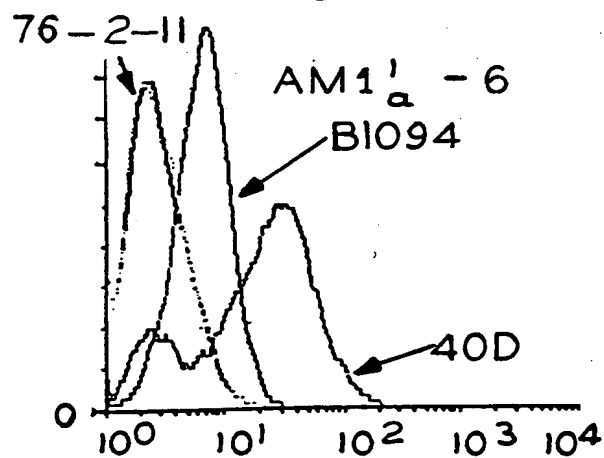


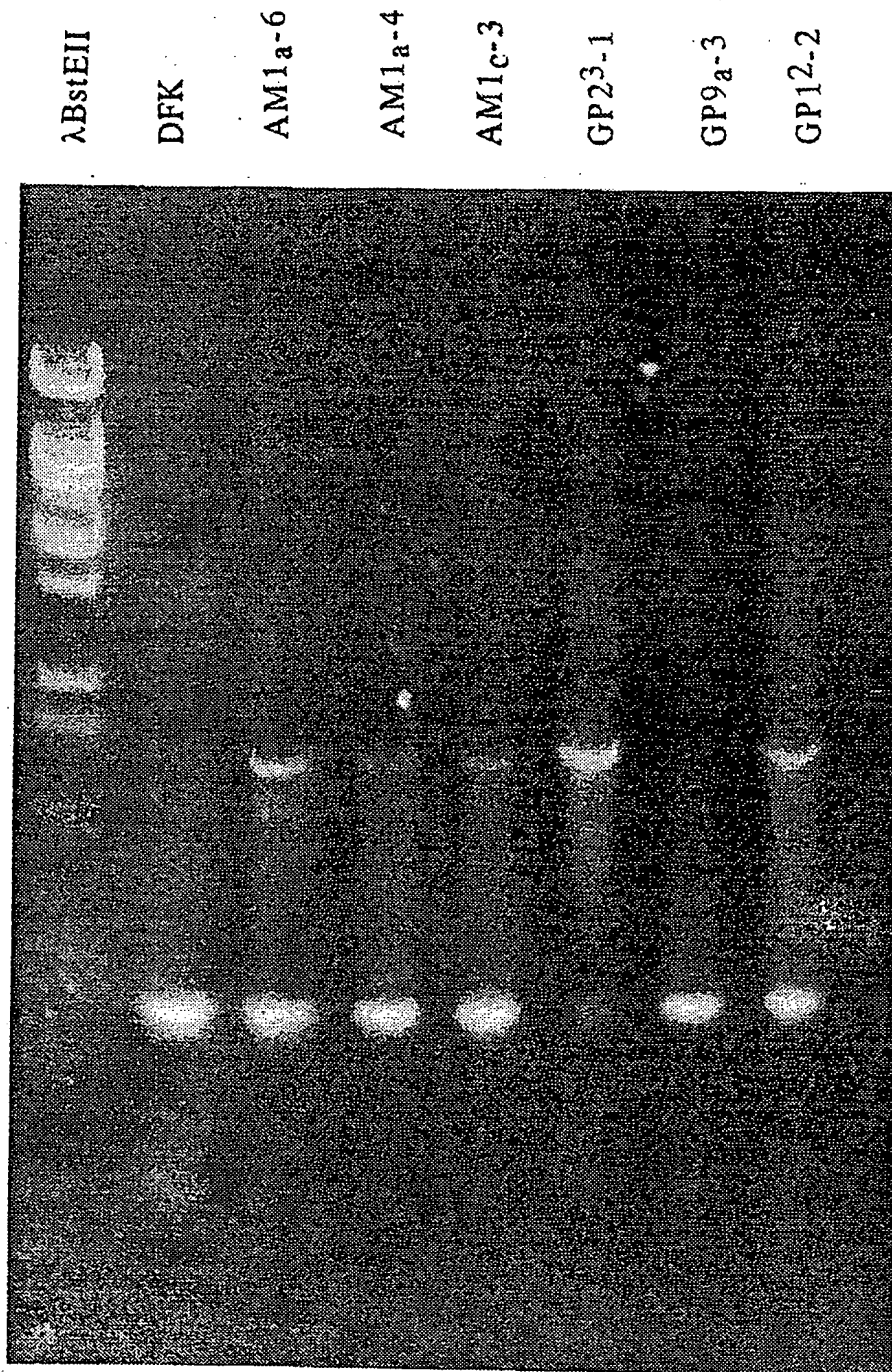
FIG. 8D



THIS PAGE BLANK (USPTO)

10/18

FIG. 9



THIS PAGE INTENTIONALLY LEFT BLANK

11/18

GP1^{2.2}
GP2³⁻¹
AM1^{1a-4}
AM1^{1c-3}
AM1^{1a-6}
λ BstEII

FIG. 10A

Probe DRβ

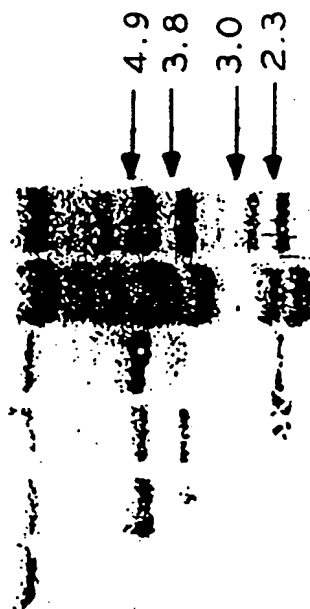
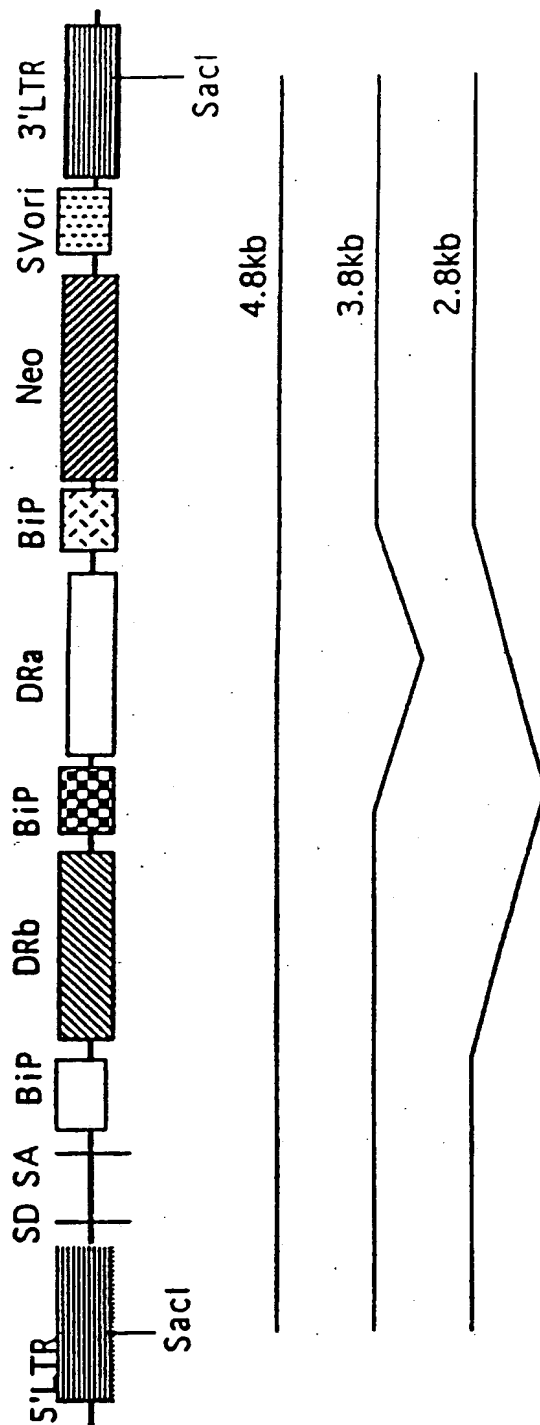


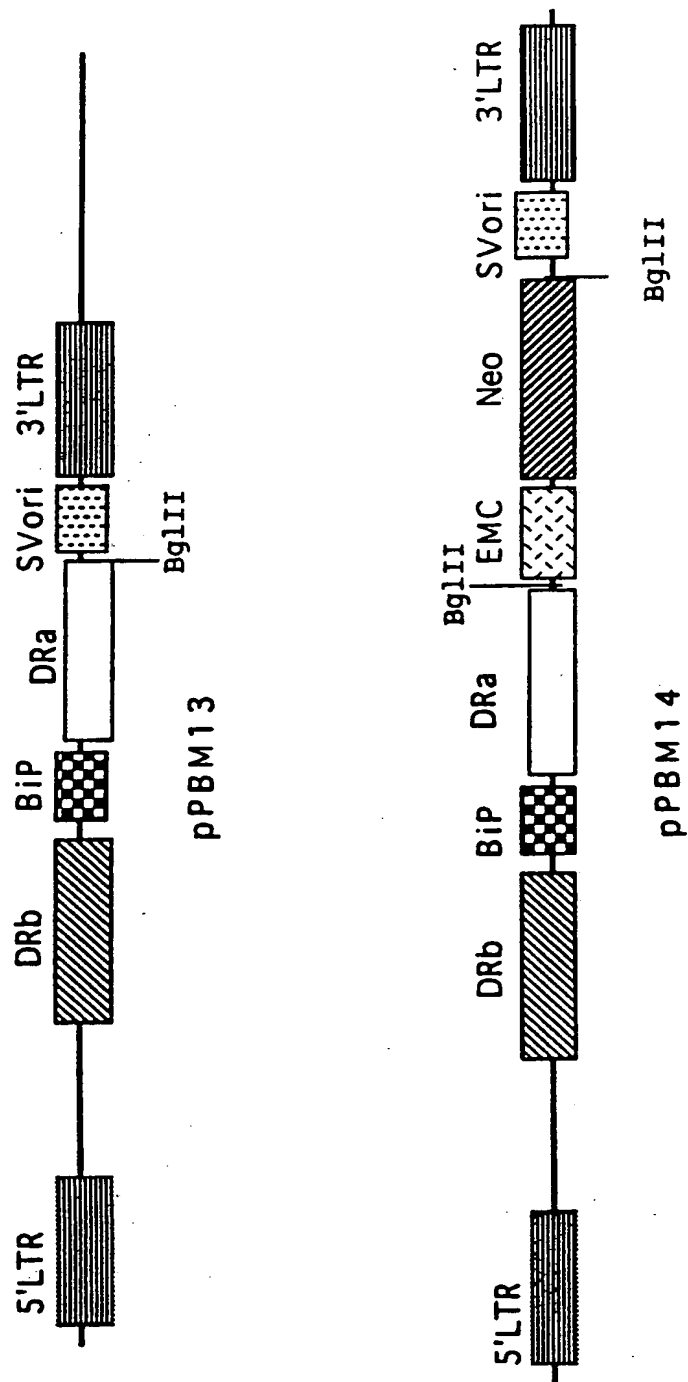
FIG. 10B



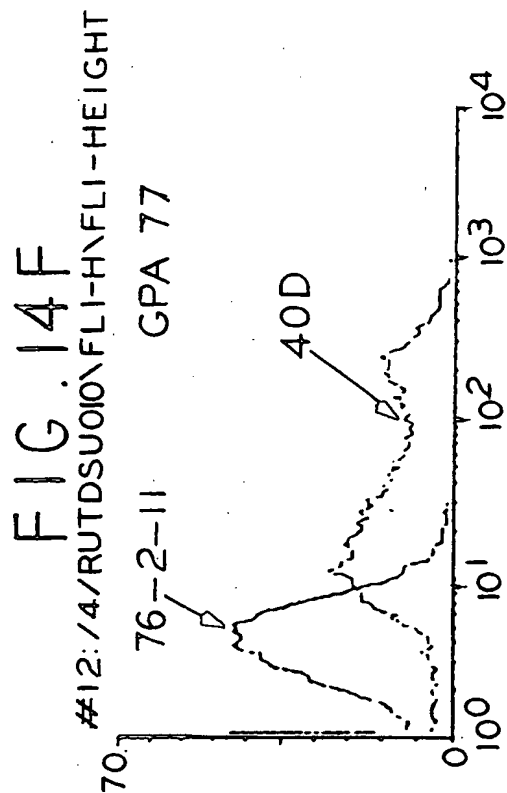
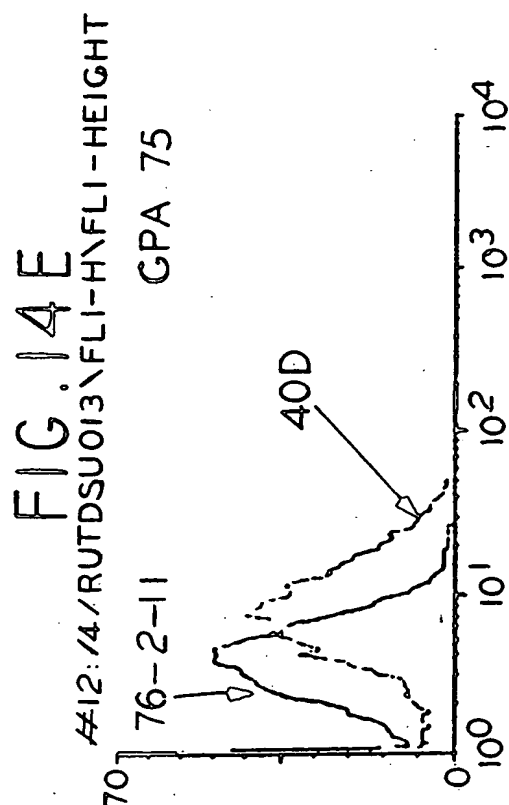
THIS PAGE BLANK (USPTO)

12/18

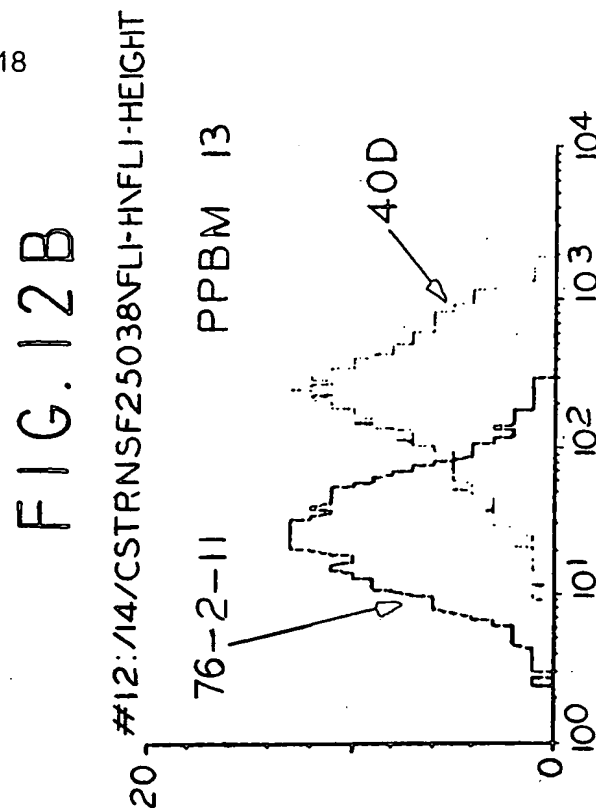
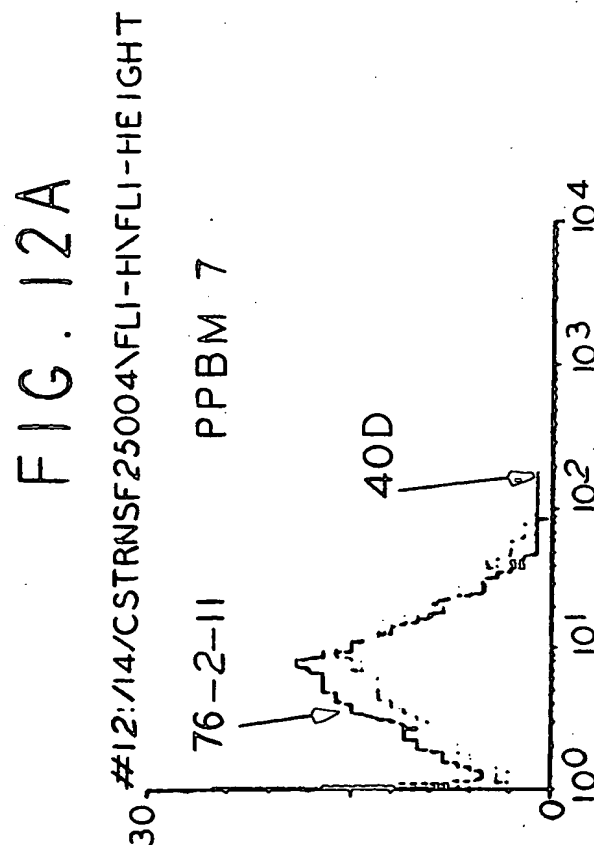
FIG. 11



THIS PAGE BLANK (USP)



13/18



THIS PAGE BLANK (US77)

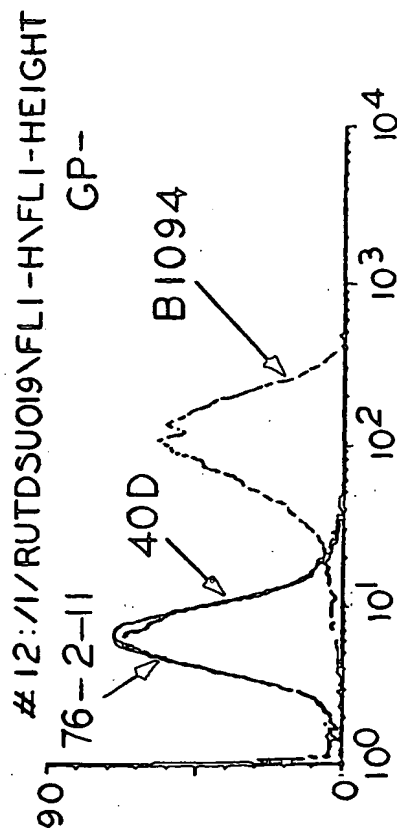


FIG. 13A

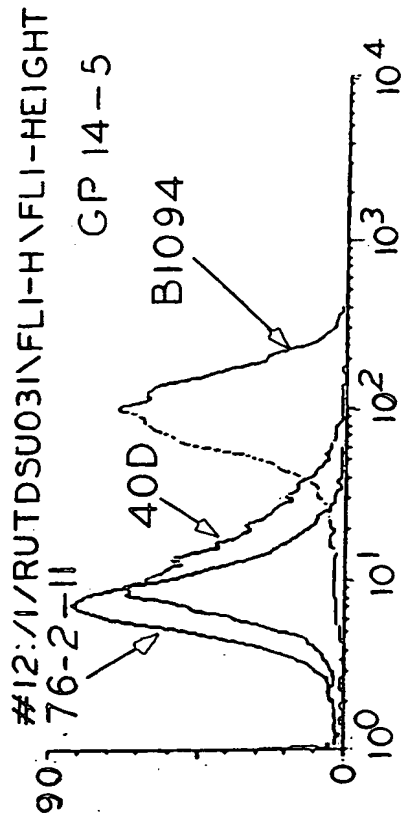


FIG. 13B

SUBSTITUTE SHEET (RULE 26)

14/18

FIG. 13C

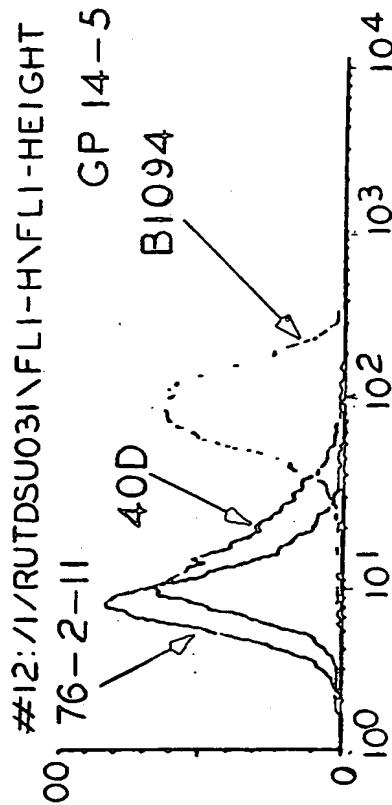
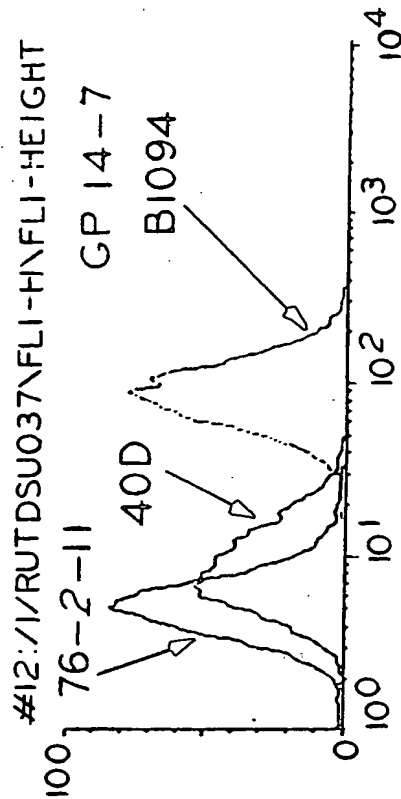


FIG. 13D



THIS PAGE BLANK (USPTO)

FIG. 14A

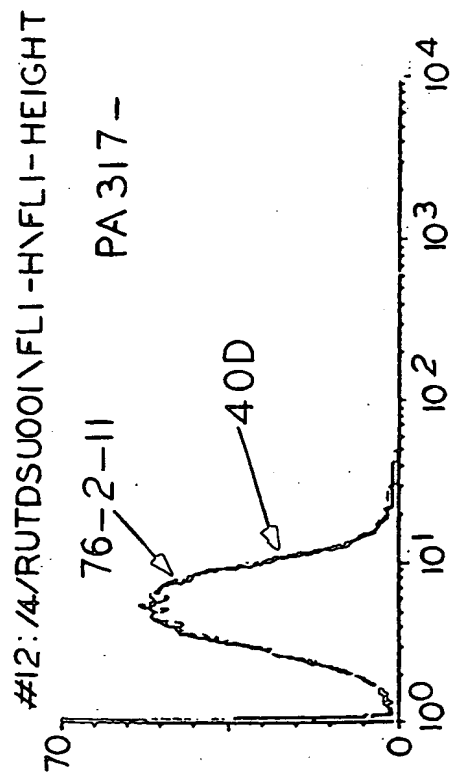


FIG. 14B

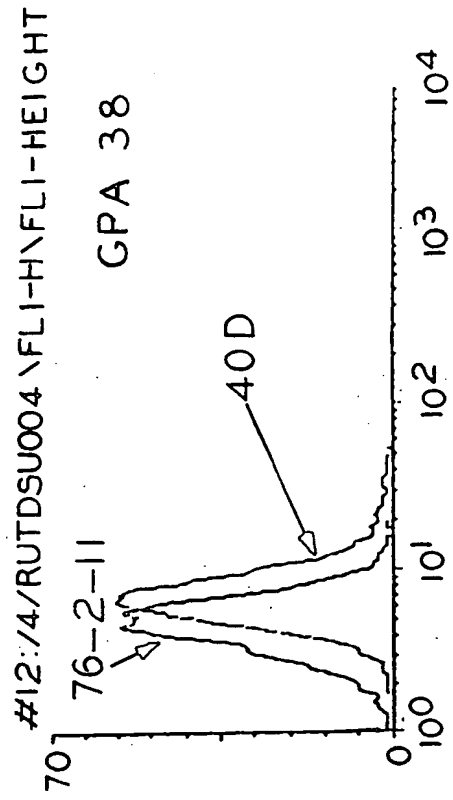


FIG. 14C

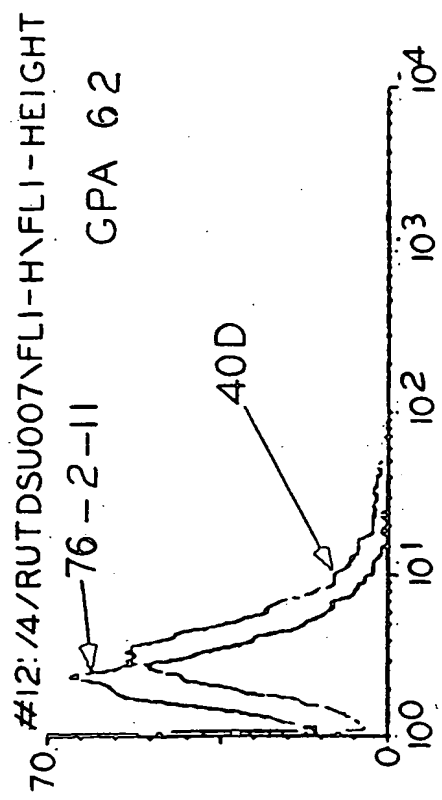
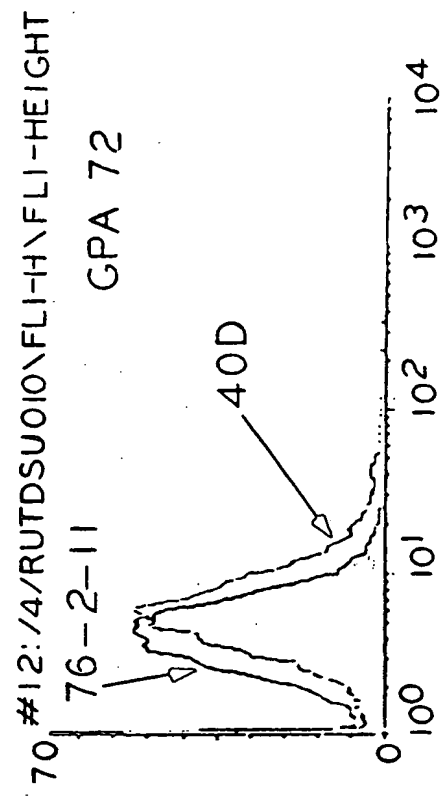


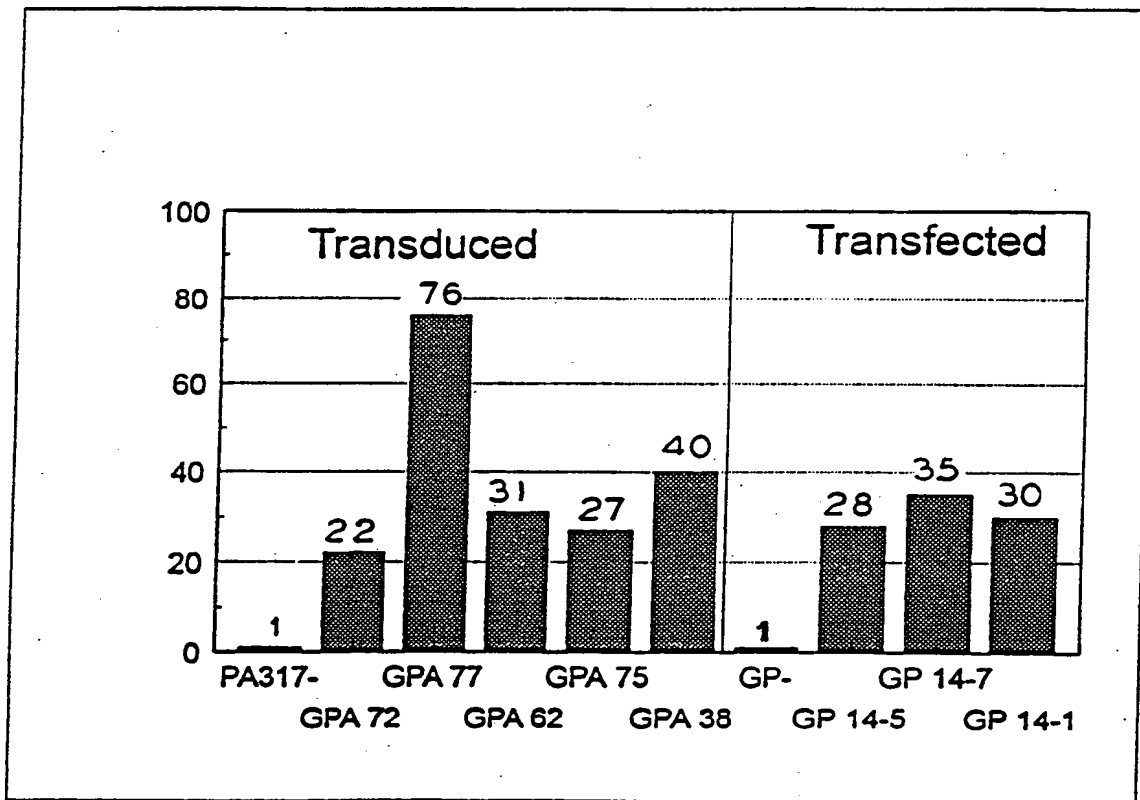
FIG. 14D



THIS PAGE BLANK (USPTO)

16/18

FIG. 15

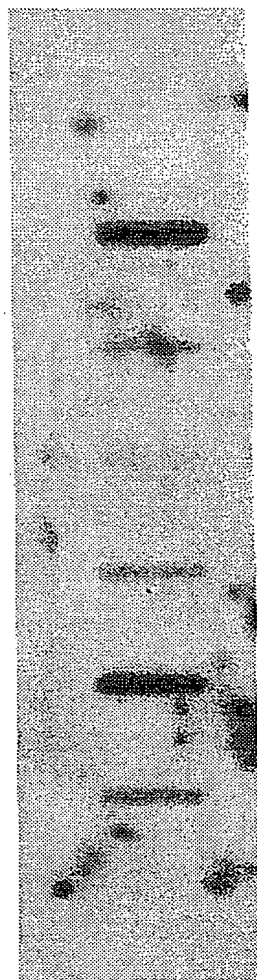


SUBSTITUTE SHEET (RULE 26)

THIS PAGE BLANK (USPTO)

17/18

FIG. 16A



PA317-

GPA 77

GPA 75

GPA 72

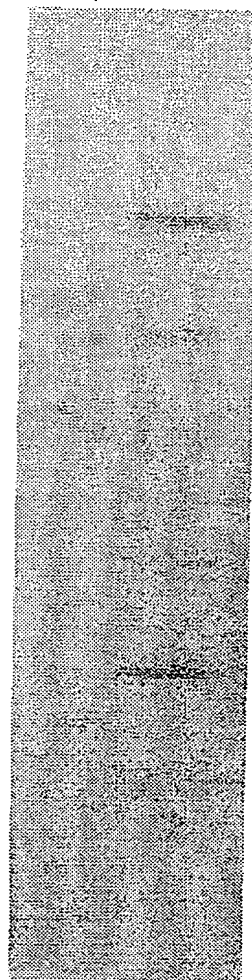
GPA 62

GPA 24

GPA 19

DFK

FIG. 16B



PA317-

GPA 77

GPA 75

GPA 72

GPA 62

GPA 24

GPA 19

DFK

SUBSTITUTE SHEET (RULE 26)

THIS PAGE BLANK (USPTO)

18/18

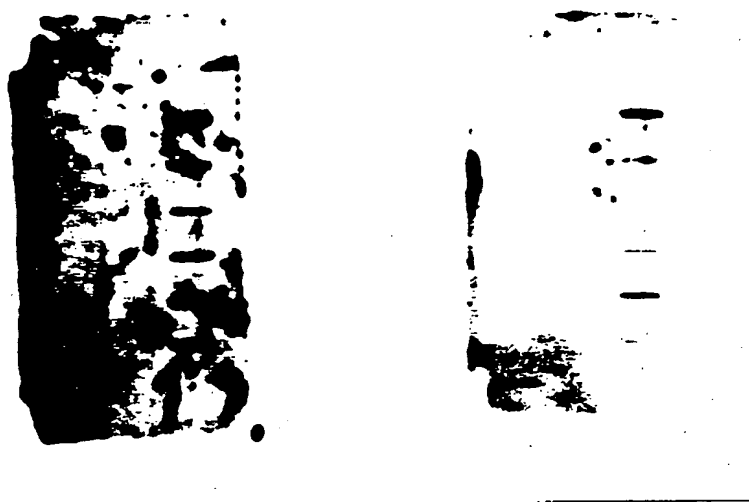


FIGURE 16

THIS PAGE BLANK (USPTO)